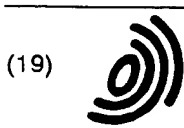


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## Remarks:

The applicant has subsequently filed a sequence  
listing and declared, that it includes no new matter.

## (54) Genes encoding truncated cellulases and their use

(57) Alkalophilic and thermophilic cellulases having  
high stability to elevated temperatures and pH have  
been isolated from an organism of unknown species,  
which most closely resembles those in the *Caldicellulo-  
sirutorgenus* and which has been called by us, Tok7B.  
1, These cellulases have been cloned and expressed in  
a recombinant system, so that they can be produced in

quantity. These are particularly useful in treating cellu-  
losic materials including cotton-containing fabrics, as  
detergent additives, and in aqueous compositions. We  
also provide genomic DNA which can be used in recom-  
binant expression vectors and expression systems to  
produce enhanced alkali and/or temperature stability  
properties in cellulases other than those specifically de-  
scribed.

Figure 2.

Blast sequence homology search with the identified N-terminal peptides shows  
the proteins have homology with Families 9 & 10 from Glycosyl hydrolases. Areas of  
homology between sequenced N-termini are shown in black backgrounds with white  
lettering.

Peptide No.	Amino-terminal amino acid sequence	Glycosyl Hydrolase Family based on amino acid homology comparisons
B1	AATNYGEALQXAIMFYEFXA	Glycosyl hydrolase Family 9
B3	AATNYGEALQ	
B5	AATNYGEALQ	
B6	QATNY	
B2	APDWSIPSLWESKYND	Glycosyl hydrolase Family 10
B4	APDWSIPSLW	

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## Description

**A FIELD OF THE INVENTION**

[0001] The present invention is directed to improved methods for treating cellulosic materials, including cotton-containing fabrics and non-cotton containing cellulose fabrics with novel truncated cellulase enzymes. In addition, this invention relates to novel truncated cellulase enzymes which exhibit cellulase activity, DNA constructs encoding the enzymes, cellulolytic agents comprising the enzymes, and detergent and water purifying or conditioning compositions containing the enzymes. In particular, this invention provides thermophilic cellulases isolated from a thermophilic anaerobic bacterial strain found in New Zealand. The cellulase genes from this organism are identified and sequenced, and the cellulases expressed from this bacterium are shown to be particularly useful in the abrasion of denim, and in the manufacture of clothing having a "stone wash" look. Most importantly, the cellulases of this invention possess unexpected proteolytic and chemical stability, as well as thermal and pH stability in hot alkaline solutions, thereby rendering them important to as laundry detergent additives in many industrial and home washing applications.

**B BACKGROUND OF THE INVENTION**

[0002] During or shortly after their manufacture, cotton-containing fabrics can be treated with cellulase enzymes in order to impart desirable properties to the fabric. For example, in the textile industry, cellulase has been used to improve the feel and/or appearance of cotton-containing fabrics, to remove surface fibers from cotton-containing knits, for imparting a "stone washed appearance to cotton-containing denims and the like.

[0003] Clothing made from cellulose fabric, such as cotton denim, is stiff in texture due to the presence of sizing compositions used to ease manufacturing, handling and assembling of clothing items. It typically has a fresh dark dyed appearance. One desirable characteristic of indigo-dyed cloth is the alteration of dyed threads with white threads, which give denim a white on blue appearance.

[0004] After a period of extended wear and laundering, the clothing items, particularly denim, can develop in the clothing panels and on the seams, localized areas of variation in the form of a lightening, in the depth and density of color. In addition, a general fading of the clothes, some pucker in the seams and some wrinkling in the fabric panels can often appear. Additionally, after laundering, sizing is substantially removed from the fabric resulting in a softer feel. In recent years such a distressed or "stonewashed" look, particularly in denim clothing has become very desirable to a substantial proportion of the public.

[0005] Previous methods for producing the distressed look included stonewashing of a clothing item or items in a large tub with pumice stones having a particle size of about 1 by 1 inches and with smaller pumice particles generated by the abrasive nature of the process. Typically the clothing item is tumbled with the pumice while wet for a sufficient period such that the pumice abrades the fabric to produce in the fabric panels, localized abraded areas of lighter color and similar lightened areas in the seams. Additionally the pumice softens the fabric and produces a fuzzy surface similar to that produced by the extended wear and laundering of the fabric. This method also enhances the desired white on blue contrast described above.

[0006] The use of pumice stones has several disadvantages, including overload damage to the machine motors, mechanical damage to transport mechanisms and washing drums, environmental waste problems from the grit produced and high labor costs associated with the manual removal of the stones from the pockets of the garments.

[0007] In view of the problems associated with pumice stones in stonewashing, cellulase solutions are used as a replacement for the pumice stones under agitating and cascading conditions, i. e., in a rotary drum washing machine, to impart a "stonewashed" appearance to the denim.

[0008] Cellulases are enzymes which hydrolyze cellulose ( $\beta$ -1,4-D-glucan linkages) and produce as primary products glucose, cellobiose, cello-oligosaccharides and the like. Cellulases are produced by a number of microorganisms and comprise several different enzyme classifications including those identified as exo-cellobiohydrolases (CBH), endoglucanases (EG), and  $\beta$ -glucosidases (BG). Enzymes within these classifications can be separated into individual components. The complete cellulase system comprising CBH, EG, and BG components synergistically act to convert crystalline cellulose to glucose.

[0009] A problem with the use of complete cellulase compositions from previously described microorganism sources for stonewashing dyed denim is the incomplete removal of colorant caused by redeposition or "backstaining" of some of the dye back onto the cloth during the stonewashing process. In the case of denim fabric, this causes recoloration of the blue threads and blue coloration of the white threads, resulting in less contrast between the blue and white threads and abrasion points (i.e., a blue on blue look rather than the preferred white on blue). This redeposition is objectionable to some users.

[0010] Some cellulases are used commercially even though they result in backstaining because of their higher activity in denim material. Either high specific activity or a high level of purity results in a higher degree of abrasion in a signif-

icantly shorter processing time and therefore is preferable to commercial denim processors.

[0011] Attempts to reduce the amount of redeposition of dye included the addition of extra chemicals or enzymes, such as surfactants, proteases, or other agents, into the cellulase wash to help disperse the loosened dye. In addition, processors have used less active whole cellulase, along with extra washings. However this results in additional chemical costs and longer processing times. Finally the use of enzymes and stones together leave the processor with all the problems caused by the use of the stones alone. Accordingly, it would be desirable to find a method to prevent redeposition of colorant during stonewashing with cellulases.

[0012] There have been previous attempts to prevent backstaining. Patent WO 92/06221 of Genencor pertains to backstaining and indicates that the cellulose biohydrolase (CBH) found in fungal cellulases is largely responsible for strength loss of the fabric and that a 5 to 1 ratio of endoglucanase to CBH is desirable. WO 96/23928, also to Genencor, relates to use of a truncated cellulase core enzyme. Both of these references emphasize the use of buffers to stabilize the cellulase solution in the wash environment. In the art it is recognized that cellulase activity is pH dependent. Most cellulases will exhibit cellulolytic activity within an acidic to neutral pH range, and the pH of an unbuffered cellulase solution could be outside the range required for cellulolytic activity. This can be undesirable and requires the addition of reagents to lower the pH of the denim following the wash cycle increasing the processing expense.

[0013] Applications of cellulases for textile processing and in commercial detergents demand proteins which are stable under highly alkaline conditions in the presence of surfactants as well as elevated temperatures.

#### **C BRIEF DESCRIPTION OF THE INVENTION**

[0014] Microorganisms from New Zealand hot springs are a recognized potential source of alkalophilic and thermophilic enzymes. We have examined numerous of these microorganisms isolated from thermal pools for their cellulase activity under alkaline conditions. The approach used was to grow the isolated bacterial cultures on cotton in order to enrich for strains that contain cellulase activity. Selected strains were grown on a larger scale and culture supernatants were then individually screened for the desired stone-wash effect. A particular strain of unknown species, but most closely resembles those in the *Caldicellulosiruptor* genus and which has been called by us, Tok7B.1, was identified from this testing. Further investigation resulted in the discovery, in accord with this invention, of six different glycosidase containing genes, designated A through F, which were identified and sequenced. These genes, or gene fragments, were selected for cellulase activity, cloned and expressed. The expressed proteins, especially those designated E1, E1/2, B5, B4/5, and E3/B5 were purified and characterized. These enzymes were shown to have alkaline activity profiles with maximal activity near pH 8.0. These proteins were tested in the textile processing applications including stone washing, and anti-staining or anti-graying, as well as other applications using alkaline pH and/or elevated temperatures, and demonstrated excellent properties in these applications. These highly active cellulase proteins, the DNA encoding these cellulase genes, and recombinant production methods and means for such production of the highly active cellulases are all provided by the invention.

[0015] This invention demonstrates that intact gene products are not required or necessarily desirable for use in many textile processing applications, and that the stability and functionality of these proteins can be varied dramatically by selective combination different genetic fragments, thereby enhancing the activity of the novel proteins herein claimed. The stability enhancing gene fragments can also be expressed with other cellulase genes to confer the improved thermal or high alkaline stability on previously described cellulase proteins.

#### **D SUMMARY OF THE INVENTION**

[0016] This invention describes thermophilic bacterial genes that encode multidomain genes containing combinations of cellulase, xylanase or cellobiohydrolase activities. Truncated forms of these genes have demonstrated useful stonewash and detergent application activities with cotton cloth. Specific oligonucleotide sequences were identified that when used as PCR primers were shown to amplify genetic sequences that, encode a series of protein domains containing glycohyrolase, thermal stabilizing and cellulose binding activities. A specific protein domain designated CelE2 was shown to function as a thermal stabilizing domain. The addition of this domain to an endoglucanase increased the thermostability by 25C. This activity could be widely applicable for enhancing the thermal stability of other genes.

[0017] The genes were obtained from the thermophilic obligate anaerobic bacterium by PCR amplification of the genomic DNA. The synthetic oligonucleotide primer sequences used for the gene amplification reactions were based on either N-terminal protein sequence data, from which degenerate probes were designed, or from genomic expression library constructs that had been screened for cellulase, cellobiosidase or xylanase activities. These specific oligonucleotide probes can serve to amplify genes useful in stone washing and/or detergent applications from other unknown bacteria that have cellulase genes.

[0018] Encoded gene fragments from the amplified genes identified as having cellulase activity were expressed in

*E. coli* either singly or in combination with cellulose binding domains and/or thermal stabilizing domains. The expressed proteins were purified to homogeneity and characterized. Cotton containing cloth treated with certain of these truncated gene constructs having endoglucanase domains and/or cellulose binding domains gave a stonewash appearance, and with other endoglucanase constructs a soil antiredeposition effect.

## **E BRIEF DESCRIPTION OF DRAWINGS**

[0019] Figures 1A and 1B are a composite drawing of protein bands containing cellulase activity purified from the supernatant broth of the Tok7B.1 organism, and their N-terminal sequences.

[0020] Figure 2 shows the results of the BLAST sequence homology search with the sequenced protein N-termini.

[0021] Figure 3 is a diagram of two consensus primers TokcelA and TokcelB and their relationship to other family 9 cellulases.

[0022] Figure 4A and 4B show the genomic walking primers and the regions amplified to obtain the complete *celE* gene and flanking regions. Figure 4C depicts a restriction map and the genetic domain structure of the *celE* gene sequence, including flanking upstream and downstream sequences.

[0023] Figure 5A is a map of W2-4 and N-17 genomic DNA fragments isolated from the Tok7B.1 genome that express cellulase activity. Figure 5B depicts the genomic walking primers and the regions amplified to obtain the complete *celA* and *celB* genes. The genetic domain structure and restriction map of *celA* and *celB* is shown in Figure 5C.

[0024] Figure 6 is a complete summary of the genetic domain structure of *celA*, *celB* and *celE* genes.

[0025] Figures 7a and 7b are a map of the restriction sites and domain structure of the Tok7B.1 genes *celC*, *celD*, *celE*, *celF*, *celG* and *celH* genes. Also the genomic walking primers used to amplify and identify each of these genes and the genetic regions amplified are indicated.

[0026] Figure 8 is a diagram of the genes and gene fragments transferred into pJLA602 controlled expression plasmid vectors.

[0027] Figure 9 is a phylogenetic analysis of the Tok7B.1 organism.

[0028] Figures 10-12 are flow diagrams for construction of the expression plasmids of pMcelE-1 and pMcelE1-2.

[0029] Figure 13 is a flow diagram for construction of the expression plasmid pMcelE1-2-3.

[0030] Figure 14 is a flow diagram for construction of the expression plasmid of pcelB4-5.

[0031] Figure 14A is a flow diagram for construction of the expression plasmid of pcelE3/B5.

[0032] Figure 15 shows the sequence analysis and MALDI-TOF of the expressed cellulases.

[0033] TABLE I lists the oligonucleotide primers designed and synthesized for study of the cellulase genes in the Tok7B.1 organism.

[0034] TABLE II lists the oligonucleotides designed for PCR amplification and directional ligation of the Tok7B.1 genes into controlled expression vectors.

[0035] TABLE III shows the gene constructs expressed in *E. coli* by a T-7 promoter.

[0036] TABLE IV is a summary T-7 expressed cellulases, their pH rate profiles, thermal stabilities and effectiveness in the stonewash application.

## **F DETAILED DESCRIPTION OF THE INVENTION**

### **I DEFINITIONS**

[0037] "Cotton-containing fabric" means sewn or unsewn fabrics made of pure cotton or cotton blends including cotton woven fabrics, cotton knits, cotton denims, cotton yarns and the like. When cotton blends are employed, the amount of cotton in the fabric should be at least about 40 percent by weight cotton; preferably, more than about 60 percent by weight cotton; and most preferably, more than about 75 percent by weight cotton. When employed as blends, the companion material employed in the fabric can include one or more non-cotton fibers including synthetic fibers such as polyamide fibers (for example, nylon 6 and nylon 66), acrylic fibers (for example, polyacrylonitrile fibers), and polyester fibers (for example, polyethylene terephthalate), polyvinyl alcohol fibers (for example, Vinyon), polyvinyl chloride fibers, polyvinylidene chloride fibers, polyurethane fibers, polyurea fibers and aramide fibers.

[0038] "Cellulose containing fabric" means any cotton or non-cotton containing cellulosic fabric or cotton or non-cotton containing cellulose blend including natural cellulose and manmade cellulose (such as Jute, flax, ramie, rayon, and the like). Included under the heading of manmade cellulose containing fabrics are regenerated fabrics that are well known in the art such as rayon. Other manmade cellulose containing fabrics include chemically modified cellulose fibers (e.g., cellulose derivatized by acetate) and solvent-spun cellulose fibers (e.g. lyocell). Of course, included within the definition of cellulose containing fabric is any garment or yarn made of such materials. Similarly, "cellulose containing fabric" includes textile fibers made of such materials.

[0039] "Treating composition" means a composition comprising a truncated cellulase component which may be used

in treating a cellulose containing fabric. Such treating includes, but is not limited to, stonewashing, modifying the texture, feel and/or appearance of cellulose containing fabrics or other techniques used during manufacturing of cellulose containing fabrics. Additionally, treating within the context of this invention contemplates the removal of "dead cotton", from cellulosic fabric or fibers, i.e. immature cotton which is significantly more amorphous than mature cotton. Dead cotton is known to cause uneven dyeing. Additionally, "treating composition" means a composition comprising a truncated cellulase component which may be used in washing of a soiled manufactured cellulose containing fabric. For example, truncated cellulase may be used in a detergent composition of, washing laundry. Detergent compositions useful in accordance with the present invention include special formulations such as pre-wash, pre-soak and home-use color restoration compositions. Treating compositions may be in the form of a concentrate which requires dilution or in the form of a dilute solution or form which can be applied directly to the cellulose containing fabric.

[0040] It is Applicants' present belief that the action pattern of cellulase upon cellulose containing fabrics does not differ significantly whether used as a stonewashing composition during manufacturing or during laundering of a soiled manufactured cellulose containing fabric. Thus, improved properties such as abrasion, redeposition of dye, strength loss and improved feel conferred by a certain cellulase or mixture of cellulases are obtained in both detergent and manufacturing processes incorporating cellulase. Of course, the formulations of specific compositions for the various textile applications of cellulase, e.g., stonewashing or laundry detergent or pre-soak, may differ due to the different applications to which the respective compositions are directed, as indicated herein. However, the improvements effected by the addition of cellulase compositions will be generally consistent through each of the various textile applications.

## II PREPARATION OF TRUNCATED CELLULASE ENZYMES

[0041] The present invention relates to the use of truncated cellulases and derivatives of truncated cellulases. These enzymes are preferably prepared by recombinant methods. Additionally, truncated cellulase proteins for use in the present invention may be obtained by other art recognized means such as chemical cleavage or proteolysis of complete cellulase protein.

[0042] The invention provides recombinant cellulase proteins which are alkalophilic and thermophilic and highly active and useful in washing applications, or in any applications including textile processing in which it is desirable to break down cellulose or cellulosic materials. It further provides DNA, free from its native genomic source, which encodes the recombinant cellulase active proteins in accord with the invention. In another preferred embodiment of this invention, we also provide genomic DNA which can be used in recombinant expression vectors and expression systems to produce enhanced alkali and/or temperature stability properties in cellulases other than those specifically described.

[0043] Also provided by the invention are bacteria cells capable of producing a native cellulase in accord with the invention and from which DNA encoding cellulases in accord with the invention may be obtained. Also provided is the native cellulase purified with respect to its native origins and associated native proteins such as by having a high protein purity or even absolute purity of at least 50%, e.g. 75%.

[0044] By way of specific preferred embodiments, this invention provides the following five particularly highly active cellulase proteins: E1, E1/2, B4/5, B5, and E3/B5.

[0045] E1 has an amino acid sequence of 446 amino acids extending from amino acid position No Y39 through amino acid position No D481 as given in Seq. ID No 44, or a function equivalent analogue thereof. DNA encoding this cellulase may vary in accord with the genetic code and a specific embodiment of such a DNA sequence comprises the DNA extending from nucleotide position No 748 through nucleotide position No 2076 as given in Sequence ID No 2.

[0046] E1/2 has an amino acid sequence of 600 amino acids extending from amino acid position No Y 39 through amino acid position No G635 as given in Seq. ID No 44, or a function equivalent analogue thereof. DNA encoding this cellulase may vary in accord with the genetic code and a specific embodiment of such a DNA sequence comprises the DNA extending from nucleotide position No 748 through nucleotide position No 2538 as given in Sequence ID No 2.

[0047] B4/5 has an amino acid sequence of 645 amino acids extending from amino acid position No K635 through amino acid position No N 1426 as given in Seq. ID No 43, or a function equivalent analogue thereof. DNA encoding this cellulase may vary in accord with the genetic code and a specific embodiment of such a DNA sequence comprises the DNA extending from nucleotide position No 8601 through nucleotide position No 10532 as given in Sequence ID No 1.

[0048] B/5 has an amino acid sequence of 418 amino acids extending from amino acid position No A 1001 through amino acid position No P 1424 as given in Seq. ID No 43, or a function equivalent analogue thereof. The B-5 protein can also end at K 1425 or N 1426, to include 419 or 420 amino acids, respectively. DNA encoding this cellulase may vary in accord with the genetic code and a specific embodiment of such a DNA sequence comprises the DNA extending from nucleotide position No 9255 through nucleotide position No 10526 as given in Sequence ID No 1.

[0049] E3/B5 has an amino acid sequence of 616 amino acids, and is a hybrid protein formed from sequences taken from the E and the B portions of the native sequences. The cel B sequence is that described from amino acid position

No K635 through amino acid position No N 1426 as given in Seq. ID No 43. DNA encoding this hybrid cellulase may vary in accord with the genetic code, but a specific embodiment of such a DNA sequence comprises the DNA starting from the *celE* gene at G2659, ending at G3123, as given in Sequence ID No 2; then joined to a segment taken from the *celB* gene starting at G9153 and ending at A10,532., as given in Sequence ID No 1., or functional equivalent analogues thereof. This E3/B5 protein and its nucleotide sequence are described in Seq ID Nos 46 and 47 respectively.

[0050] As will be recognized by those skilled in the art, DNA encoding active cellulases in accord with the invention may be modified in various ways to produce such cellulases for practical usage. For example, the DNA encoding a signal sequence may be removed and replaced by the codon ATG encoding for Met at amino acid position No. 31, using known techniques. The resulting DNA which lacks a signal sequence may be used to express active cellulase in accord with the invention, more particularly in *E. coli*, which cellulase product depending on the host strain will produce a cellulase with or without Met at its N-terminus, or mixtures of such products. Similarly, the signal sequence may be replaced by known techniques with other signal sequences to improve production, particularly secretion into the production media, and/or to adapt the DNA to particular hosts for production.

[0051] The cellulase gene-containing inserts cloned and provided in accord with our invention contain all the control or regulatory sequences necessary for expression of the structural gene in bacterial hosts, particularly *Bacillus* and *E. coli* hosts. These sequences, such as promoter sequences, ribosome binding site sequences and the like may also be modified or replaced in whole or in part by other control sequences using known techniques to improve production and/or to adapt the DNA to particular hosts for production. When such a change is made, the resulting DNA sequence is deemed to involve the structural gene in sequence with heterologous DNA.

[0052] The DNA encoding an active alkalophilic and thermophilic cellulase in accord with the invention may be incorporated into a wide variety of vectors for various purposes such as replication of such DNA or expression of the structural gene or for purposes of causing incorporation of the DNA into the genome of a host cell for ultimate expression of the encoded gene. Such vectors will typically involve DNA sequences containing the DNA encoding the active cellulase recombined with other heterologous DNA. The terms heterologous DNA and the like as used herein generally refer to a DNA sequence which has a functional purpose and which is either different from the sequences in or obtained from a source other than the native Tok7B.1 DNA from which the instant gene was cloned, thereby creating a continuous sequence which is not found or associated with the cellulase gene in the native Tok7B.1 source. Examples of such functional sequences are many and include for purposes of illustration origins of replications, genes for antibiotic resistance and also various control sequences, such promoter sequences to be used for effecting expression of the structural gene itself, as well as flanking sequences suitable for causing insertion of DNA containing the gene coding sequence into a host genome. Such vectors include for illustration only those commonly referred to as plasmids and those which are viral vectors. The construction of vectors is well-known and DNA sequences of widely different origins and/or recombinations are available for such construction, such sequences also commonly called plasmids, viral vectors and the like. For example, a vector in accord with the invention and used by us can be obtained from the known plasmid pUC18 which contains the pBR 322-derived ampicillin resistance gene and origin of replication, together with a portion of the *E. coli* *lacZ'* gene (*lacZ'*) encoding the  $\alpha$ -complementation peptide.

[0053] This *lacZ'* fragment has been engineered to contain a multiple cloning site (MCS). DNA inserted into the MCS inactivates the *lacZ'* gene, providing blue/white color selection of recombinants when appropriate hosts and indicator plates are used. The complete gene or clone we obtained can be inserted or ligated into the MCS and expressed in an *E. coli* host by operation of its own native control sequences.

[0054] In general, the vectors of the invention are constructed with reference to suitability for incorporation into particular host cells, and such transformed cells are also a part of the invention. As used herein, the term "transformed" and the like means the incorporation of vector DNA into a host cell independent of the purpose in terms of replication of the recombinant gene or its expression, or both, and whether the vector DNA remains intact in the cell or its contained cellulase encoding gene is incorporated for expression into the cell genome. The vectors of the invention may be transformed into any of a variety of cell types such as bacterial cell, yeast cells, insect and mammalian cells. Preferably, the transformed cells are bacteria or yeast cells, and more preferably are gram negative bacteria such as *E. coli* or gram positive bacteria such as *Streptomyces* or *Bacillus* cells where such *Bacillus* cells are not of thermophilic source, such preferred *Bacillus* types including *Bacillus subtilis* and the like. Methods for transforming cells with vectors are generally well-known.

[0055] The invention also provides a process for producing the recombinant cellulase active proteins of the invention comprising culturing cells transformed with a recombinant expression vector of the invention comprising promoter DNA operatively controlling expression of the DNA encoding the cellulase protein. Methods of culturing such transformed cells to effect their multiplication and expression of the cellulase encoding gene of the transformed vector DNA are also well-known. Procedures for recovery of the recombinantly produced proteins are also known and may be used to obtain the cellulase of the invention in the more practical forms for use. In general, the recombinantly produced cellulase as expressed by the transformed cells may be retained within the cells and/or secreted into the culture media. When retained in quantity within the cells, the cells are lysed such as in a Waring Blender, sonifier or pressure cell to liberate

the cellulase into the culture media which is then usually treated to separate cellular debris and preferably filtered to obtain the cellulase in the resulting aqueous supernatant or filtrate. When secreted into the media, the culture liquid media or supernatant containing the cellulase is simply separated from the cells. Such filtrates and supernatants may then be used as a basis for a product for treatment of cellulosic materials, typically after concentration. Such cellulase-containing liquids may also be treated, for example by microfiltration, to separate undesired materials including lower molecular weight proteins. The resulting aqueous cellulase-containing compositions may also be treated to enhance their storage or use properties, for example, by addition of buffers to enhance stability of the cellulase. Hence, the cellulase products may be buffered between pH 5 to 10, preferably pH 7 to 9, using, for example, Tris buffer.

[0056] The cellulases of the present invention have been found to be particularly useful for additives used in the cleaning or treatment of cellulose fabrics, including cotton-containing fabrics. They exhibit high activity even at high temperatures or high pH, thereby facilitating their suitability of aqueous detergent solutions and formulations.

[0057] It will be recognized that the cellulases of the invention are obtained from a microorganism characteristic of those which are thermophilic and alkalophilic and which produce a variety of enzymes which may be similarly classified by favoring conditions encountered in natural thermally heated alkaline pools. A variety of microorganisms have been identified in such pools. The cellulases of this invention originate from a particular strain of unknown species which most closely resembles those in the *Caldicellulosiruptor* genus and which has been called by us, Tok7B.1.

### III DEPOSITS

[0058] We have under the Budapest Treaty conditions, deposited with the American Type Culture Collection at Rockville, MD, USA, a biologically pure culture of the cells indicated below, which deposits were assigned the Accession Numbers given below along with their date of deposit.

Identification and Content of Deposit	Accession No.	Deposit Date
E. coli BL21 (DE3) Cel E	ATCC 98523	August 29, 1997
E. coli DH $\alpha$ F' 1Q Cel B	ATCC 98524	August 29, 1997
Tok7B.1 bacterial strain	ATCC 202028	September 10, 1997

[0059] As will be recognized, any of the above deposits may be cultured under condition to cause expression of a cellulase of the invention in accord with the experiments described herein and such cellulase products recovered in a variety of product forms for use as also described herein. Alternatively, cultures of the deposited cells may be grown to multiple the number of copies of their contained plasmidal clones and the cellulase gene and coding sequence may be separated from the plasmids by the use of restriction enzymes, preferably by partial digest with Sau3AI, and the DNA encoding the cellulase (for example, an approx. 1.57 Kb fragment upon Sau3AI partially digest) used for a variety of purposes including production of active cellulase protein of the invention in a wide variety of other expression systems.

### IV METHODS OF TREATING CELLULOSE CONTAINING FABRIC USING TRUNCATED CELLULASE ENZYMES

[0060] As noted above, the present invention pertains to methods for treating cellulose containing fabrics with a truncated cellulase enzyme. The use of the truncated cellulase composition of this invention provides the novel and surprising result of effecting a relatively low level of dye redeposition while maintaining an equivalent level of abrasion compared to prior art cellulase treatment. Because the level of abrasion acts as an indicator of the quality and effectiveness of a particular cellulase treatment techniques, e.g., stonewashing or laundering, the use of the instant invention provides a surprisingly high quality textile treatment composition. In the laundering context, abrasion is sometimes referred to as color clarification, defuzzing or biopolishing.

[0061] The present invention specifically contemplates the use of truncated cellulase core, alone or in combination with additional cellulase components, to achieve excellent abrasion with reduced redeposition when compared to non-truncated cellulase. Additionally, naturally occurring cellulase enzymes which lack a binding domain are contemplated as within the scope of the invention. It is also contemplated that the methods of this invention will provide additional enhancements to treated cellulose containing fabric, including improvement in the feel and/or appearance of the fabric.

### A) METHODOLOGY FOR STONEWASHING WITH TRUNCATED CELLULASE COMPOSITIONS

[0062] According to one aspect of the present invention, the truncated cellulase compositions described above may be employed as a stonewashing composition. Preferably, the stonewashing composition of the instant invention com-



prises an aqueous solution which contain a an effective amount of a truncated cellulase together with other optional ingredients including, for example, a buffer, a surfactant, and a scouring agent.

**[0063]** An effective amount of truncated cellulase enzyme composition is a concentration of truncated cellulase enzyme sufficient for its intended purpose. Thus an "effective amount" of truncated cellulase in the stonewashing composition according to the present invention is that amount which will provide the desired treatment, e.g., stonewashing. The amount of truncated cellulase employed is also dependent on the equipment employed, the process parameters employed (the temperature of the truncated cellulase treatment solution, the exposure time to the cellulase solution, and the like), and the cellulase activity (e.g., a particular solution will require a lower concentration of cellulase where a more active cellulase composition is used as compared to a less active cellulase composition). The exact concentration of truncated cellulase can be readily determined by the skilled artisan based on the above factors as well as the desired result. Preferably the truncated cellulase composition is present in a concentration of from 1-1000 PPM, more preferably 10-400 PPM and most preferably 20-100 PPM total protein.

**[0064]** Optionally, a buffer is employed in the stonewashing composition such that the concentration of buffer is that which is sufficient to maintain the pH of the solution within the range wherein the employed truncated cellulase exhibits activity which, in turn, depends on the nature of the truncated cellulase employed. The exact concentration of buffer employed will depend on several factors which the skilled artisan can readily take into account. For example, in a preferred embodiment, the buffer as well as the buffer concentration are selected so as to maintain the pH of the final truncated cellulase solution within the pH range required for optimal cellulase activity. Preferably, buffer concentration in the stonewashing composition is about 0.001N or greater. Suitable buffers include, for example, citrate and acetate.

**[0065]** In addition to truncated cellulase and a buffer, the stonewashing composition may optionally contain a surfactant. Preferably, the surfactant is present in a concentration in the diluted wash mediums of greater than 100 PPM, preferably from about 200-15,000 PPM. Suitable surfactants include any surfactant compatible with the cellulase and the fabric including, for example, anionic, non-ionic and ampholytic surfactants. Suitable anionic surfactants for use herein include linear or branched alkylbenzenesulfonates; alkyl or alkenyl ether sulfates having linear or branched alkyl groups or alkenyl groups; alkyl or alkenyl sulfates; olefinsulfonates; alkanesulfonates and the like. Suitable counter ions for anionic surfactants include alkali metal ions such as sodium and potassium; alkaline earth metal ions such as calcium and magnesium; ammonium ion; and alkanolamines having 1 to 3 alkanol groups of carbon number 2 or 3. Ampholytic surfactants include quaternary ammonium salt sulfonates, and betaine-type ampholytic surfactants. Such ampholytic surfactants have both the positive and negative charged groups in the same molecule. Nonionic surfactants generally comprise polyoxyalkylene ethers, as well as higher fatty acid alkanolamides or alkylene oxide adduct thereof, and fatty acid glycerine monoesters. Mixtures of surfactants can also be employed in manners known in the art.

**[0066]** In a preferred embodiment, a concentrated stonewashing composition can be prepared for use in the methods described herein. Such concentrates would contain concentrated amounts of the truncated cellulase composition described above, buffer and surfactant, preferably in an aqueous solution. When so formulated, the stonewashing concentrate can readily be diluted with water so as to quickly and accurately prepare stonewashing compositions according to the present invention and having the requisite concentration of these additives.

**[0067]** Preferably, such concentrates will comprise from about 0.1 to about 50 weight percent of a cellulase composition described above (protein); from about 0.1 to about 80 weight percent buffer; from about 0 to about 50 weight percent surfactant, with the balance being water. When aqueous concentrates are formulated, these concentrates can be diluted so as to arrive at the requisite concentration of the components in the truncated cellulase solution as indicated above. As is readily apparent, such stonewashing concentrates will permit facile formulation of the truncated cellulase solutions as well as permit feasible transportation of the concentration to the location where it will be used. The stonewashing concentrate can be in any art recognized form, for example, liquid, emulsion, gel, or paste. Such forms are well known to the skilled artisan.

**[0068]** Other materials can also be used with or placed in the stonewashing composition of the present invention as desired, including stones, pumice, fillers, solvents, enzyme activators, and other anti-redeposition agents.

**[0069]** The cellulose containing fabric is contacted with the stonewashing composition containing an effective amount of the truncated cellulase enzyme or derivative by intermingling the treating composition with the stonewashing composition, and thus bringing the truncated cellulase enzyme into proximity with the fabric. For example, if the treating composition is an aqueous solution, the fabric may be directly soaked in the solution. Similarly, where the stonewashing composition is a concentrate, the concentrate is diluted into a water bath with the cellulose containing fabric. When the stonewashing composition is in a solid form, for example a pre-wash gel or solid stick, the stonewashing composition may be contacted by directly applying the composition to the fabric or to the wash liquor.

**[0070]** The cellulose containing fabric is incubated with the stonewashing solution under conditions effective to allow the enzymatic action to confer a stonewashed appearance to the cellulose containing fabric. For example, during stonewashing, the pH, liquor ratio, temperature and reaction time may be adjusted to optimize the conditions under which the stonewashing composition acts. "Effective conditions" necessarily refers to the pH, liquor ratio, and temperature which allow the truncated cellulase enzyme to react efficiently with cellulose containing fabric. The reaction con-



ditions for truncated cellulase core, and thus the conditions effective for the stonewashing compositions of the present invention, are substantially similar to well known methods used with corresponding non-truncated cellulases. Similarly, where a mixture of truncated and non-truncated cellulase is utilized, the conditions should be optimized similar to where a similar combination may have been used. Accordingly, it is within the skill of those in the art to maximize conditions for using the stonewashing compositions according to the present invention.

[0071] The liquor ratios during stonewashing, i.e., the ratio of weight of stonewashing composition solution (i.e., the wash liquor) to the weight of fabric, employed herein is generally an amount sufficient to achieve the desired stonewashing effect in the denim fabric and is dependent upon the process used. Preferably, the liquor ratios are from about 4:1 to about 50:1; more preferably from 5:1 to about 20:1, and most preferably from about 10:1 to about 15:1. Reaction temperatures during stonewashing with the present stonewashing compositions are governed by two competing factors. Firstly, higher temperatures generally correspond to enhanced reaction kinetics, i.e., faster reactions, which permit reduced reaction times as compared to reaction times required at lower temperatures. Accordingly, reaction temperatures are generally at least about 10°C and greater. Secondly, cellulase is a protein which loses activity beyond a given reaction temperature which temperature is dependent on the nature of the cellulase used. Thus, if the reaction temperature is permitted to go too high, then the cellulolytic activity is lost as a result of the denaturing of the cellulase. As a result, the maximum reaction temperatures employed herein are generally about 65°C. In view of the above, reaction temperatures are generally from about 30°C to about 65°C; preferably, from about 35°C to about 60°C; and more preferably, from about 35°C to about 55°C.

[0072] Reaction times are dependent on the specific conditions under which the stonewashing occurs. For example, pH, temperature and concentration of truncated cellulase will all effect the optimal reaction time. Generally, reaction times are from about 5 minutes to about 5 hours, and preferably from about 10 minutes to about 3 hours and, more preferably, from about 20 minutes to about 1 hour.

[0073] Cellulose containing fabrics treated in the stonewashing methods described above using truncated cellulase compositions according to the present invention show reduced redeposition of dye as compared to the same cellulose containing fabrics treated in the same manner with an non-truncated cellulase composition.

#### **B) METHODOLOGY FOR TREATING CELLULOSE CONTAINING FABRICS WITH A DETERGENT COMPOSITION COMPRISING TRUNCATED CELLULASE ENZYME**

[0074] According to the present invention, the truncated cellulase composition described above may be employed in detergent compositions. The detergent compositions according to the present invention are useful as pre-wash compositions, pre-soak compositions, or for detergent cleaning during the regular wash cycle. Preferably, the detergent composition which can be dry mixed or in an aqueous liquid formulation, of the present invention comprises an effective amount of truncated cellulase, and a surfactant, and optionally include other ingredients and additives commonly employed in detergent formulations. An effective amount of truncated cellulase employed in the detergent compositions of this invention is an amount sufficient to impart improved anti-graying, anti-staining, anti-backstaining, or anti-soil deposition of cotton or cellulosic containing fabrics. Preferably, the truncated cellulase employed is in a concentration of about 0.001% to about 25%, more preferably, about 0.02% to about 10% by weight percent of detergent.

[0075] The specific concentration of truncated cellulase enzyme employed in the detergent composition is preferably selected so that upon dilution into a wash medium, the concentration of truncated cellulase enzyme is in a range of about 0.1 to about 1000 PPM, preferably from about 0.2 PPM to about 500 PPM, and most preferably from about 0.5 PPM to about 250 PPM total protein. Thus, the specific amount of truncated cellulase enzyme employed in the detergent composition will depend on the extent to which the detergent will be diluted upon addition to water so as to form a wash solution.

[0076] At lower concentrations of truncated cellulase enzyme, i.e., concentrations of truncated enzyme lower than 20 PPM, the decreased backstaining or redeposition with equivalent surface fiber abrasion when compared to prior art compositions will become evident after repeated washings. At higher concentrations, i.e., concentrations of truncated cellulase enzymes of greater than 40 PPM, the decreased backstaining with equivalent surface fiber removal will become evident after a single wash.

[0077] This invention is illustrated by the following procedures and examples.

[0078] Applications of cellulases for textile processing and in commercial detergents demand proteins that are stable under conditions of alkaline pH and elevated temperatures.

#### **V EXAMPLES**

##### **Isolation of cellulase secreting microorganisms from alkaline thermal pools**

[0079] To identify thermal stable glycolytic proteins, microorganisms were isolated from the water and sediment

samples taken from geothermal pools in the central volcanic region of New Zealand's North Island. The criteria for the pools sampled were temperatures of at least 50° C and pH values of greater than 6.0. A total of twenty samples were collected from geothermal pools that met the criteria. Each of the samples contained a complex mixture of microorganisms. In order to enrich the samples for microorganisms that expressed cellulase genes with desired cellulase activity 1 mL volumes of the collected sample were inoculated into 10 mL of 2/1 medium in Hungate tubes containing either amorphous cellulose (7g/L) or unbleached cotton fabric (approximately 1 cm square) as cellulose substrate, at pH 7.0 and pH 8.5. These tubes were incubated at 70° C and the cultures viewed microscopically after 4 days. The enrichment strategy was based on the assumption that the presence of the cellulosic fibers would induce expression of the cellulase genes in the microorganisms, and that those microorganisms would flourish under these conditions. From this collection of organisms the anaerobic, cellulase producer, Tok7B.1 was isolated from a water/sediment sample taken from Tokaanu Pool 7, situated in the central volcanic region of the North Island, New Zealand. The pH and temperature of this particular pool at the time of sampling were pH 7.5 and 60° C, respectively.

[0080] The 2/1 medium and amorphous cellulose, pH 7.0 proved the most favorable for the growth of the anaerobic rods from the Tok7B.1 sample, and after further subculturing, PAHBAH (p-hydroxybenzoic acid hydrazide) assays (Lever, 1973) on the concentrated supernatant confirmed the presence of cellulase-producing organisms. The substrate for these PAHBAH assays was 0.2% carboxymethyl cellulose (low viscosity) in 100 mM Taps buffer pH 8.8 at 20° C.

[0081] A pure culture of Tok7B.1 was obtained using a version of the Roll Tube method described by Hungate (1969). Serial dilutions of the positive cultures were made in Hungate tubes containing the growth medium + 18 g/l agar. The agar/culture mixture was solidified around the inside of the sealed tube by rolling in a flat dish containing iced water. Tubes were incubated at 70° C and single colonies removed aseptically using a Pasteur pipette with the tip bent a right angle. A plug of agar was placed in liquid medium and the cells released by crushing against the side of the tube. Positive identification of a cellulase producer was again confirmed by PAHBAH assays of the culture supernatants. To detect secreted cellulases supernatants from the cultures were concentrated approximately ten fold prior to being assayed for cellulase activity using CMCase assay.

[0082] The Tok7B.1 cellulases were identified in a secondary screening assay that served to evaluate the biostone washing effectiveness of the cellulases secreted into the sample supernatant. Each of the cultures selected for screening was fermented in sufficient quantity and the supernatants concentrated in order to provide sufficient activity for the biostone wash testing, approximately 10,000 CMCase units. The supernatants were tested in a 2L drum denim assay at equivalent levels of CMCase activity. The cellulases were tested under the following conditions; pH 7.0 for 60 minutes at 50° C using 135g of blue denim samples were washed for 1h at pH 7.0. The light reflectance value on the blue denim cloth and from a swatch of white cloth included in the wash were determined by measuring the level of denim abrasion and backstaining, respectively. Blue denim samples that demonstrated a reflectance value of above 15 and a dose dependent effect with increasing concentrations of fermentation supernatants were considered to contain candidate cellulases. White cloth swatches that have a reflectance of below 4 were acceptable for backstaining. Based on these tests the Tok7B.1 organism was found to produce the most effective cellulases, giving the highest abrasion with the lowest backstaining of the samples tested.

#### Strategy for Identifying Industrially useful cellulases

[0083] Our strategy was to identify industrially useful cellulases secreted from the Tok7B.1 organism, then to identify the individual genes responsible for that activity. The following steps were carried out to clone the individual genes, express these genes in an intermediary expression system and test the individual cellulases in the application. The first step in the strategy was to identify the individual proteins secreted by the Tok7B.1 bacterium. Identification of the individual cellulases secreted by the bacterium was important because identification of the genes effective in the application would limit the number of cellulase genes and gene constructs that would have to be expressed and tested.

#### Cellulase Nomenclature

[0084] Genes and genetic constructs are designated in small letters and are italicized, for example the genes that encode the CelE proteins are designated *celE*. Conversely proteins are designated by capitalizing the first letter and are not italicized, for example, CelE1. The Tok7B.1 cellulase genetic domains are designated in Figure 6, and one should be careful not to confuse these with the protein designations shown in the third column of Table III. For example the CelE1 protein is comprised of the second genetic domain in the *celE* gene.

#### Identification of N-terminal Sequences of Tok7B.1 cellulases

[0085] The culture supernatant from the Tok7B.1 strain was chromatographed on a Mono-S column (Pharmacia) at

pH 5.0 in 10 mM sodium acetate buffer at a flow rate of 1 ml/min. The bound proteins were eluted with a 30 ml linear gradient of NaCl from 0-250 mM. Each of the fractions collected was assayed for CMCase activity. 73% of the total CMCase activity was collected into fractions and 27% of the activity was found in the column flow-through. The proteins from fractions that demonstrated CMCase activity were electrophoresed on an 8% SDS polyacrylamide gel. Protein bands in fractions containing cellulase activity could be observed in a Coomassie-stained 8% SDS polyacrylamide gel. The cellulase activity of these bands was confirmed in part by overlaying the SDS polyacrylamide gel with an agarose gel containing carboxymethyl cellulose (CMC). Cellulases not denatured by the SDS degrade the CMC in the agarose gel. These areas of degraded CMC can be identified by staining with Congo Red using the methods of Beguin (1983) and Mackenzie and Williams (1984). Proteins of interest were blotted from the SDS-PAGE gel onto an Immobilon membrane and then the amino terminal sequences determined by Edman degradation (Matsudaria, 1987). The sequences determined for each of the individual bands are shown in a composite drawing (Figure 1). CMCase activity that was not captured on the Mono-S column was subsequently buffer-exchanged into 12 mM Tris buffer pH 9.0, chromatographed on a Q sepharose column (1.5 x 6 cm), and eluted with a 30 mL linear gradient of 0-250 mM NaCl. Fractions that contained CMCase activity were electrophoresed on an 8% SDS PAGE and gave a protein band with identical apparent molecular weight and N-terminal sequence to the B5 band (Figure 1) previously identified from the S-sepharose column.

[0086] The N-termini of each of these proteins was determined by Edman degradation. Only two different amino acid sequences were determined from the six proteins N-terminally sequenced. The N-terminus of the *celE* gene product was homologous with four of the proteins identified and the N-terminus of the *celB* gene product wash homologous with the two remaining protein bands. The amino acid sequence information served first to identify the genes that were expressing the cellulases useful for the applications. Second, the N-terminal sequences were compared with the protein sequences in GenBank using the Basic Linear Alignment Search Technique (BLAST. Jauris, et al., 1990). This confirmed that the two proteins sequenced belonged to the glycosyl-hydrolase family. The *celB* gene product has an amino-terminal sequence which shares significant homology with a general class of xylan degrading enzymes referred to as Family F beta-glycanases (Gilkes et al., 1991) or Family 10 glycosyl-hydrolase (Henrisatt, 1991). The *CelE* gene product shares homology with family E beta-glycanases/Family 9 glycosyl-hydrolases.

#### Strategy for the cloning of the cellulase genes

[0087] Our strategy for identifying the Tok7B.1 glycolytic genes was to employ two approaches simultaneously. 1) Polymerase chain reaction (PCR) with primers based on the sequence information obtained from the BLAST search was used PCR to amplify gene sequences from the Tok7B.1 genomic DNA preparations. 2) An expression library of the Tok7B.1 genomic DNA was constructed and screened for the expression of proteins able to degrade CMC.

#### Methods and Prior Art

[0088] Agarose gel electrophoresis, plasmid isolation, M13 mp10 single stranded DNA isolation, use of DNA modifying enzymes and *E. coli* transformation were performed as described by Sambrook et al. (1989).

#### Genomic DNA Preparation

[0089] Tok7B.1 genomic DNA was prepared from a cell culture which had been grown under anaerobic conditions for 1-2 days without shaking at 70°C in 2/1 media. Cells were harvested from the growth media by centrifugation at 5000 rpm for 10 minutes, then resuspended in 50 ml TES buffer before a second centrifugation step. Cell pellets were then resuspended in 5ml 50mM Tris pH 8.0, mixed with 374µl 0.5M EDTA and incubated for 20 minutes at 37°C. After the addition of 550µl freshly prepared lysozyme (10mg/ml), the mixture was incubated at 70°C for 20 minutes, mixed with 250µl *Streptomyces griseus* protease (40mg/ml) and 310µl 10% SDS, then left to incubate overnight at 70°C. After allowing the lysed cells to cool to room temperature, the resulting clear solution was phenol extracted 2-5 times until no material could be seen to partition at the interface. The remaining volume of the sample was estimated and a 1/10 volume of 3M Sodium acetate was added and mixed, then 2.5 volumes of 95-100% ethanol gently layered onto the top of the sample. DNA could be seen as a stringy white precipitate at the interface of the two liquids and could be removed by spooling onto the end of a Pasteur pipette. Spooled DNA was transferred into a 1.5ml microcentrifuge tube and washed in 70% ethanol before air drying for 1-3 hours. The resulting DNA pellet was resuspended in TE buffer and left overnight to fully dissolve. All genomic DNA preparations were stored at 4°C.

[0090] Isolation of the Tok7B.1 *celE* gene using consensus PCR and Genomic walking PCR The Tok7B.1 *celE* gene, gene product CelE, was identified by amino-terminal sequencing of cellulolytic peptides secreted by Tok7B.1 (Figure 2). The *celE* gene codes for a family 9 glycosyl hydrolase based on comparison to translated gene sequences in the GenBank database. The CelE peptide sequence shared highest similarity to family 9 glycosyl hydrolases from

other thermophilic Clostridial microorganisms. Homology alignments of family 9 genes indicated that it would be possible to design consensus oligonucleotide primers which would bind to DNA coding for clusters of highly conserved amino acids found in all thermophilic Clostridial family 9 glycosyl hydrolases. These consensus primers could then be used in PCR to amplify family 9 glycosyl hydrolase genes from Tok7B.1. Two primers were designed, the first, tokcela, bound to DNA coding for the peptide sequence QKAIMFYEF, and tokcelr, which bound in the reverse orientation (with respect to the gene sequence) to DNA coding for the peptide sequence DYNAGFVGAL (Figure 3).

[0091] The tokcela and tokcelr primers were used to amplify an approximately 1300bp PCR product from Tok7B.1. This product was ligated into M13 mpIO (Messing, 1983), transformed in *E. coli* strain JM101 and plated to give individual recombinant plaques. In order to test whether the PCR product was generated from a single gene, or from multiple genes, PCR product was reamplified from individual plaques using the M13 forward and reverse primers then mapped by restriction digestion with *Tsp509I*. A total of 12 individual PCR products were restriction mapped and all showed identical restriction patterns. Six of these PCR products were sequenced and all showed identical DNA sequence. This data indicated that all cloned PCR products were amplified from a single family 9 glycosyl hydrolase gene present on the genome of Tok7B.1. In order to obtain the complete *celE* gene sequence, new PCR primers were designed to allow genomic walking upstream and downstream of the region covered by the 1300bp PCR product (Figure 4A). Standard subcloning and DNA sequencing techniques were used to obtain 6416bp of DNA sequence containing the entire *celE* gene sequence plus flanking upstream and downstream sequence (Figure 4B). The complete DNA sequence and translated peptide sequence of the *celE* gene is given in Sequence #2.

#### Genomic Library construction and screening

[0092] Genomic DNA from Tok7B.1 was partially digested with the restriction endonuclease *Tsp509I* to give DNA fragments in the size range of 6-8kb. These fragments were then ligated into *XhoI*-digested  $\lambda$ ZapII (Stratagene, 11011 North Torrey Pines Road, La Jolla, CA 92037, USA) then packaged and plated according to protocols supplied by Stratagene. Individual plaque isolates shown to contain genomic inserts using the blue/white lacZ complementation system present in  $\lambda$ ZapII were replated, and a total of 1600 genomic insert containing plaques were screened for thermophilic cellulase and xylanase activity at 70°C using the substrate overlay method of Teather and Wood (1982). Cellulase activity was detected using the soluble cellulose derivative carboxymethyl cellulose (CMC). Plaques were also screened for cellobiohydrolase activity using the chromogenic substrate methylumbelliferyl cellobioside (MUC) as described by Saul et al. (1990).

[0093] Two positive  $\lambda$ ZapII plaques, designated W2-4 and N17, were isolated which expressed thermophilic xylanase and/or cellulase activity (Figure 5A). These recombinant phage were converted to Bluescript SK- plasmids using the standard Exassist excision procedure described by Stratagene. Each plasmid was restriction mapped using a range of restriction endonucleases. Common restriction endonuclease digestion patterns indicated that W2-4 and N17 contained common overlapping DNA from the same region of the Tok7B.1 genome (Figure 5A).

#### DNA sequencing and sequence analysis of the Tok7B.1 *celB* and *celA* genes

[0094] The recombinant DNA from W2-4 and N17 was partially sequenced by creating simple plasmid deletions using known restriction sites within the plasmid insert (Gibbs, et al. 1991). Initial DNA sequence homology comparison data indicated a gene coding for a multidomain enzyme with a xylanase and a cellulase domain and several internal cellulase binding domains (CBD). The Genomic DNA contained by W2-4 was sequenced in full, and portions of N17, by subcloning and sequencing internal restriction fragments and using synthesized DNA oligonucleotide primers (primers are listed in Table I). Analysis of the complete sequence of W2-4 showed that the DNA contained a complete gene, *celB*, coding for a nine-domain protein designated CelB. The 3'-portion of a further gene was observed to lie upstream of the *celB* gene. This gene, designated *celA*, shared at least 1 domain in common with the *celB* gene. The complete coding sequence of *celA* was obtained using Genomic Walking PCR (GW-PCR) as described by Morris et al. (1994). Representative GW-PCR products spanning the region of the *celA* gene are depicted in Figure 5B. The complete DNA sequence containing the *celA* and *celB* genes is depicted in Figure 5C, with each gene shown according to its translated domain structure. The complete DNA sequence and translated peptide sequence of the *celA* and *celB* genes is given in Sequence # 1. The translated product of the *celB* gene matches perfectly with two amino-terminal sequences obtained for native cellulolytic peptides secreted by Tok7B.1 (Figure 2, peptides B2 and B4), implying that the *celB* gene expresses one of the major cellulases secreted by Tok7B.1.

[0095] A complete summary of the protein domain structures of CelA and CelB is given in Figure 6.

[0096] The complete *celE* gene was observed to code for a large multidomain-multicatalytic enzyme with a putative length of 1751 amino-acids (unprocessed) and is composed of at least 10 discrete functional domains based on homology comparisons (figure 6). The family 9 glycosyl hydrolase domain is the amino-terminal domain of the full length CelE, while the central domains of CelE (domains 4-9, figure 6) are virtually identical to the central domains of CelB

(domains 3-8, figure 6), the only exception being the relative lengths of each PT-linker. The carboxy-terminal domain of CelE (domain 10, figure 6) is homologous to the carboxy-terminal endoglucanase domain (family 44 glycosyl hydrolase) of ManA from *C. saccharolyticus*. This domain can degrade xylan as well as carboxymethylcellulose (Gibbs et al. 1991) and activity assays have shown that the carboxy-terminal domain of Tok7B.1 CelE is also an endoglucanase with weak xylanase activity.

#### **Identification of further Tok7B.1 cellulase genes using GW-PCR, *celC* and *celH***

[0097] In the process of obtaining the complete coding sequence of the Tok7B.1 *celE* gene further ORFs were identified upstream of this gene. Homology comparisons indicated that these genes also coded for cellulolytic enzymes. GW-PCR was used to obtain DNA sequence from upstream of the *celE* (figure 7A.) Two further genes were identified in this way. Both of these genes, designated *celC* and *celH*, code for multidomain, multicatalytic proteins, with the same general structure as CelA, CelB and CelE. As the DNA sequence obtained was not contiguous, long-template PCR (Expand Long template PCR System, Boehringer Mannheim, Australia Pty. Ltd.) was used to amplify DNA between the sequenced regions to confirm that they were contiguous (figure 7A). Approximately 13500bp of genomic DNA upstream of the *celE* gene was partially sequenced.

#### **Identification of *celF* and *celG***

[0098] During the isolation of the complete *celA* gene sequence the primer N17a was used as a genomic walking primer. A number of PCR products were obtained which did not match DNA sequence already obtained for the *celB* and *celA* genes. It was clear from these results that the N17a primer was CelB. Upstream of this second xylanase domain a further gene was identified coding for an enzyme with a carboxy-terminal family 48 glycosyl hydrolase domain. These genes were designated *celF* and *celG* respectively (figure 7B). Oligonucleotide primers specific to the carboxy-terminal end of the *celG* gene and the amino-terminal end of the *celF* gene were synthesized and used in combination with oligonucleotide PCRs which bound to DNA coding for the CBDs found in *celA*, *celB*, *celE*, *celC* and *celH*. The amplification of PCR products indicated that *celG* and *celF* coded for the proteins with the same basic domain structure of the other Tok7B.1 cellulolytic genes. The amino-terminal domain of *celG* was not identified, the carboxy-terminal of *celF* was identified as a family 48 glycosyl hydrolase with high homology to the carboxy-terminal domains of *celG* and *celC*.

#### **Transfer of Tok7B.1 genes into controlled-expression plasmid vectors**

[0099] To facilitate the transfer of Tok7B.1 cellulase genes into controlled-expression plasmid vector the general method of Gibbs et al. (1991) was used. PCR was used to amplify full length cellulase genes (and portions of cellulase genes). Oligonucleotide primers corresponding to each end of the gene were engineered to contain restriction sites allowing directional ligation of restriction digested PCR product into plasmid multiple cloning sites. Table II. lists the oligonucleotides designed for PCR amplification and directional ligation of the various Tok7B.1 genes into controlled expression vectors. Each primer contains one or more restriction endonuclease site(s) to facilitate ligation of PCR product into plasmid vector predigested with the same restriction enzyme, resulting in an in-frame gene fusion between each thermophilic gene and a signal peptide sequence encoded on the vector. The various genes and gene fragments transfer into pJLA602 by this method are shown in Figure 8.

#### **Phylogenetic analysis of Tok7B.1**

[0100] The 16S SSU rRNA gene was isolated using PCR. A PCR product was generated using oligonucleotide primers designed to amplify the 16S SSU rRNA gene from all known prokaryotic species. An approximately 1800bp PCR fragment was obtained which was cloned into M13 mpIO in the forward and reverse orientation, and sequenced (Seq #3). The SSU rRNA gene sequence obtained was compared to all genes in the GenBank database. Close homologs of the Tok7B.1 SSU rRNA gene were aligned using the GCG multiple alignment software 'Pileup'. Resulting aligned sequence files were subsequently analyzed using parsimony methods (Swofford, 1993). Figure 9 shows the phylogenetic position of Tok7B.1 amongst cluster D of thermophilic Clostridia (Rainey et al., 1993).

#### **Cloning of individual genes into an *E. coli* expression vector**

[0101] From the *celE* and *celB* genes a number of new truncated genes containing either individual cellulase catalytic domains Cel EI or catalytic domains connected to cellulose binding domains by linker sequences, Cel E1/2, CelE1/2/3 and CelB4/5 have been constructed (Table III). Each of the genes have been individually expressed in *E. coli* using

the bacteriophage T-7 RNA polymerase/promoter system (Studier and Moffatt, 1986).

#### Expression Cloning of the CelE Domains D2

[0102] The N-terminal CelE endoglucanase catalytic domain (Figure 6) and the first cellulose-binding domain (CBD) (Figure 6) were used to construct expression plasmids pcelEI and pcelEI/2 respectively. These *celE* gene domains were obtained from the M13-mp10 clones, M13celE1 and M13celE1/2. The first step in the cloning process was the PCR amplification of domain 2 or domains 2 plus 3 of the *celE* gene from Tok7B.1 genomic DNA (Figure 10). Unique restriction endoglucanase sites were introduced by the PCR primers at the 5' and 3' ends of the gene fragments. An *SphI* site was incorporated at the 5' end of the native gene at the predicted translational start site, which encodes the translational ATG start codon, and *BglII* sites were incorporated at the 3' ends of the specific gene domains at convenient locations. Translational stop codons were introduced just upstream of the *BglII* sites. The PCR fragments were blunt end ligated directly into *SmaI* digested M13mp10 vector, (Messing, 1983) to give the clones M13-celE1 and M13-celE1/2 (Figure 11).

[0103] Using the pET9a vector (Novagen) *E. coli* expression plasmids were constructed. The plasmid utilizes the T7 Polymerase promoter for gene expression, (Studier, et al., 1990). An intermediate construct was employed to facilitate the cloning process. The *celE* 1/2/3 gene was amplified using PCR, the forward direction primer tokcbdf, and the reverse direction primer tokcel (Figure 11). The forward primer, tokcbdf introduces a *NdeI* site at the 5' end of the mature *celE* gene and thereby encodes the translational ATG start codon. The introduction of the *NdeI* restriction site changed the first two amino acids encoded in the mature sequence from GT to AA Table III. The reverse PCR primer, tokcel, was homologous to the native gene sequence at the *NdeI* site in CBD domain 3. The PCR fragment was digested with *NdeI* and gel-purified with silica gel technology using a Qiaex II gel extraction kit from Qiagen Inc. The fragment was ligated into the *NdeI* site of the pET9a vector (Figure 12). The resulting plasmid, pMcelE-*NdeI*, was digested with *PstI* and *BamHI* and the vector fragment was isolated from the digest by agarose gel electrophoresis and silica gel purification. The M13-celE1 and M13-celE1/2 clones were digested with *PstI* and *BglII* and the resulting *celE* gene fragments, *celE*1 and *celE*1/2, were isolated from the digest by agarose gel electrophoresis and silica gel purified (Figure 12). The fragments were ligated to the *PstI*-*BamHI* digested pcelE-*NdeI* plasmid to form the final clones, pMcelEI and pMcelEI/2 (Figure 12). Both the *BglII* and *BamHI* restriction enzymes produce compatible sticky ends but these sites are lost upon ligation.

#### Expression Cloning of the CelE D2/3/4/5

[0104] The pcelE1/2/3 plasmid encodes the first catalytic domain of the *celE* gene plus the first two cellulose-binding domains D3 and D5 (Table III) in a pET9a expression vector. The catalytic domain D2 and CBD D3 used in the construction of the pcelE1/2/3 expression plasmid was obtained from the pcelEI/2 plasmid. The second cellulose-binding domain D5 was obtained from the pRR9 plasmid (Figure 8). The construction of the final plasmid required a three-way ligation that is outlined in Figure 13.

[0105] The entire native *celE* gene was amplified by PCR from genomic Tok7B.1 DNA using the tocelef forward primer and the tokcelr3 reverse primer Table II. The PCR primers contained an *SphI* site in the forward primer, which introduces the ATG translational start codon, and a *Sall* site in the reverse primer. The PCR fragment was digested with *SphI* and *Sall* and cloned into the *SphI* and *Sall* sites of the polylinker of the *E. coli* expression vector pJLA602, to produce the pRR9 plasmid (Figure 8). To obtain the gene fragment encoding domains 4 and 5 for ligation with the pcelE1/2 plasmid, the region from the *NcoI* site in D3 through D5 was PCR amplified from the pRR9 plasmid (Figure 13). Tokcelef, the forward primer, was homologous to the *celE* sequence at the *NcoI* site and the tokcelebamr reverse primer was homologous to the end of D5, the second CBD in *celE* and introduced a *BamHI* cloning site. This PCR fragment was digested with *NcoI* and *BamHI* and purified. The *celE* fragment from D2 to the 5' end of D3 at the *NcoI* site was isolated from the plasmid pcelEI/2 (Figure 13). The plasmid was digested with *NdeI* and *NcoI* and the *celE* fragment was isolated from the vector fragment by gel electrophoresis and silica gel technology. The vector, pET9a, was digested with *NdeI* and *BamHI* and purified by gel electrophoresis and silica gel technology. The two *celE* fragments were ligated to the pET9a expression vector in a three-part ligation to produce the pcelEI/2/3 plasmid (Figure 13).

#### Expression cloning of CelB4/5

[0106] A plasmid that expressed the CelB4/5 protein of the Tok7B.1 *celB* gene was constructed in the *E. coli* expression vector, pET9a, as described below. Domains 7, 8 and 9 containing a CBD and catalytic domain were PCR amplified from the Tok7B.1 genomic DNA using primers tokcbdf and tokcelbr. These primers incorporated into the PCR fragment a unique 5' *NdeI* site by the forward primer and a unique 3' *BamHI* site (Figure 8). The fragment was digested with *NdeI* and *BamHI* and ligated into the *NdeI* and *BamHI* digested pJLA602 expression vector to produce the pRR6

plasmid (Figure 14). The pRR6 plasmid was digested with *NdeI* and *BamHI* and the *celB* gene was purified from the vector fragment by gel electrophoresis and silica gel technology. The pET9a vector was digested with *NdeI* and *BamHI* and purified by gel electrophoresis and silica gel technology. The two fragments were ligated together to produce the pcelB4/5 plasmid (Figure 14).

#### **Expression Cloning of CelB3/4/5**

[0107] The CBDs of the *celE* gene, domains 4 & 5, (Figure 8) are very homologous to the CBDs of the *celB* gene, domains 3 & 4, (Figure 8). Also, the two CBDs within the genes are very homologous to each other. This homology is useful for the construction of the pcelB3/4/5 construct in the *E. Coli* expression vector pET9a. A homologous region of domain 3 of the *celB* gene is cloned from the *celE* gene construct. This is done by taking advantage of a *BglII* site in each of the homologous *celE* CBD domains 4 & 5. This *BglII* fragment is isolated by restriction digest from the *celE* construct pRR10 which encodes domains 3,4,5, & 6 of the *celE* gene, Figure 8, in the pJLA602 expression vector. This *BglII* fragment contains the 3' portion of *celE* Domain 3 and the 5' portion of *celE* Domain 4. This *BglII* fragment is ligated into the *BglII* site of Domain 4, the CBD, of pcelB4/5. The resulting plasmid is pcelB3/4/5.

#### **Expression cloning of CelE3/B5**

[0108] This clone is constructed in the *E. Coli* expression vector pET9a. Domain 3 of the *celE* gene is PCR amplified from pcelE1/2/3. The forward and reverse primers incorporated into the PCR fragment provide unique 5' *NdeI* and 3' *BstEII* sites. The PCR fragment is digested with *NdeI* and *BstEII* and ligated to the pcelB4/5 vector which is digested with *NdeI* and *BstEII* and gel purified (Figure 14A). The *NdeI* and *BstEII* digest of the pcelB4/5 results in the removal of the native *celB* CBD as well as 29 amino acids from the PT linker.

#### **Fermentation of the *E. coli* expressing cloned cellulase genes**

[0109] The pcel E1, pcel E1/2, pcel E1/2/3, pcelB4/5, pcelB3/4/5 and pcel E3/B5 expression plasmids were transformed into *E. coli* DE3-BL21 (Stratagene Corp.). Transformants were grown at 37° C to an OD600 of 1.0 in 250 mL of L-broth containing 50 µg/ml Kanamycin. The 250 ml of L-broth was then used to inoculate a 20 L Chemap fermentor containing 12 liters of media. The fermentation media consisted of 12 g/L of tryptone, 24 g/L yeast extract, KH<sub>2</sub>PO<sub>4</sub> 2.3 g/L, 12.5 g/L K<sub>2</sub>HPO<sub>4</sub>, 1 mL/L Antifoam 289 (Sigma), 4g/L glycerol, 1 mL/L 1.0 M MgSO<sub>4</sub> · 7H<sub>2</sub>O and 50 µg/mL Kanamycin. The transformants were grown at 37° C to an OD600 of approximately 12 and then expression was induced by the addition of IPTG at a concentration of 95 mg/L. After a 3h induction the cells were harvested by centrifugation in 500ml bottles at 7,000 x g for 10 min. A typical yield from a 12-L fermentation was 300 g of wet cell paste. Cell pellets were then frozen at -80° C prior to lysis and purification of the recombinant proteins.

#### **Purification of the Cel E1 and Cel E1/2 Cellulases**

[0110] The *E. coli* fermentation cell pellets were thawed by resuspending the frozen cells in two volumes of 20 mM Tris buffer pH 8.0. The cells were homogenized with a Virtis Virtishear 1200 for 20 min., then lysed by one passage through a Microfluidizer (Microfluidics Corp.) at a pressure of 9600 psi. The lysate was centrifuged at 43,000 x g for 30 min. The pellet was discarded and the supernatant was combined with sufficient ammonium sulfate to make a 1 molar solution. The ammonium sulfate solution was stored overnight at 4° C then centrifuged at 15,000 x g for 20 minutes. The supernatant was then chromatographed on phenyl sepharose. The column (5 x 10 cm) was washed with 10 mM Tris pH 8.0, 1.0 M ammonium sulfate. After the column effluent had an A280 of less than 0.1 AU, the protein was eluted with a 300 mL linear gradient from 1.0 M to 0 M ammonium sulfate. This column eluent was used in the application testing. Each of the constructs tested in the application was electrophoresed on a 12% polyacrylamide gel and then blotted to an Immobilon membrane and N-terminally sequenced. Figure 16 shows the expected N-terminal sequenced versus the sequence found upon Edman degradation.

#### **Purification of the CelB5 and CelB4/5 Cellulases**

[0111] When the Cel B4/5 protein purification described below is carried out in the presence of a protease inhibitor cocktail consisting of phenylmethyl sulfonyl fluoride, EDTA and Aprotinin, the full length protein, CelB4/5, consisting of the CBD, PT linker region and catalytic domain is purified. However, in the absence of the protease cocktail, the linker region is cleaved to yield the Cel B5 endoglucanase domain alone, without the CBD or PT linker domains.

[0112] For purification of the CelB4/5, 280 g of cells expressing celB4/5 were thawed in three volumes of 10 mM Tris, pH 7.0 in the presence of the protease cocktail described above. The thawed cells were virtisheared for 20 min.



then lysed as before by a single pass on the Microfluidizer. The lysate was centrifuged for 10 min. at 3,500 x g. The resulting supernatant (820ml) was heated in a 50° C water bath for 10 minutes, then centrifuged for 20 minutes at 3,000 x g. Sufficient (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> was added to give a 20% saturated solution, the solution was centrifuged for 30 min. at 3,000 x g and the pellet discarded. More (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> was added to the supernatant until the solution was 35% saturated, the solution was centrifuged for 30 min. at 3,000 x g and the supernatant discarded. The pellet was resuspended in 10 mM Tris pH 8.0, 0.5 mM EDTA, 1 mM Aprotinin.

[0113] The solution was chromatographed on a 430 ml DEAE column (5 cm x 20 cm) and eluted with a two-step NaCl gradient. Step one of the elution profile was 0 to 150 mM NaCl wash in 300 ml, step two was a wash of 150 mM to 260 mM NaCl linear gradient in 1200 ml. The CMCase activity eluted between 750-950 ml and gave 1.5 g of CelB4/5 protein.

[0114] CelB5 was purified in an identical manner except the only protease inhibitor added to the cell lysate supernatant was 1mM PMSF. CelB5 eluted in an identical manner from the DEAE column. The total protein purified was 1g from about 280 g of cells.

#### Purification of CelB3/4/5

[0115] 400g of frozen cells are thawed in 800 ml of 10 mM Tris, pH 8.0, 0.5 mM EDTA. The cells are lysed by one pass through the Microfluidizer at 12,000 psi. The lysed sample is then centrifuged at 7,800 x g for 50 min. To the supernatant (950 ml) is added slowly 100.7 g of ((NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>) to give a 20% saturated solution. The solution is stirred overnight for 12h at 4° C. The precipitated proteins were removed by centrifugation for 30 minutes at 14,000 x g. The remaining supernatant is brought up to 40% (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> and left to stir for 48 h at 4° C. The precipitate is pelleted by centrifugation for 30 min at 15,000 x g. The pellet is resuspended in 20 mM Mes pH 6.0. The conductivity is reduced to less than 3 ohms/cm<sup>2</sup> by diafiltration using a 30kD Filtron membrane. The dialysate is centrifuged to remove any precipitate and chromatographed on S-sepharose (10 cm x 6 cm) and eluted with a linear salt gradient from 0.1M to 0.35M. Fractions containing activity of greater than 200 units/mL are pooled. The final pool contains 720 mg of protein which is approximately 52 % pure as determined by densitometry scanning of a Coomassie stained 12% SDS PAGE of the pool.

#### Purification of CelE3/B5

[0116] 400 gm of E. coli DE3-B121 are thawed in 10 mM Tris, pH 8.0, 0.5 mM EDTA. The cells are lysed by passage through the microfluidizer at 12,000 psi. The precipitate is removed by centrifugation of the lysate for 30 min at 8,000 x g. To the supernatant is then added solid (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> to give a 20 % saturated solution. The precipitate is removed by centrifugation at 14,000 x g for 30 min and the supernatant was loaded on a phenyl sepharose column 6 cm x 10cm. The protein is eluted with a 2L reverse linear gradient from 1 M to 0 M (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> in lysis buffer. The bulk of the activity is collected in three fractions. Each of the fraction contains 250 ml. Each of the fraction is analyzed for the activity.

[0117] The conductivity is reduced to less than 3 ohms/cm<sup>2</sup> by diafiltration using a 30kD Filtron membrane with a 10 mM Imidazole pH 7.0. The dialysate is chromatographed on S-sepharose (10 cm x 6 cm) and eluted with a linear salt gradient from 0 M to 0.23 M. Fractions containing activity of greater than 250 units/mL are pooled. The final pool contains 720 mg of protein which is approximately 86.9 % pure as determined by densitometry scanning of a Coomassie stained 12% SDS PAGE of the pool.

#### pH rate profiles of purified Cellulases

[0118] The pH rate profiles and thermostability of the cellulases were determined. These data serve to define the pH extremes at which an enzyme could be used in an application. Cellulases were assayed at 50° C for the determination of the pH rate profiles. The catalyzed rates of reaction at each pH are expressed as fractions of the fastest observed rate. This is calculated by dividing the rate of reaction at each pH by the highest reaction rate observed at any pH, the highest reaction rate is therefore plotted as 1.0. The CMC substrate and buffer in each case was made with an appropriate buffer for each pH being tested. The following buffers were employed for each of the assays, at pH 3.0 sodium tartrate (25 mM), pH 4.0 sodium tartrate (50 mM), pH 5.0 sodium acetate (50 mM), pH 7.0 sodium phosphate (50 mM), pH 9.0 glycine (50 mM), pH 10.0 glycine (50 mM), pH 11.0 CAPS (50 mM), pH 12.0 sodium phosphate (50 mM). 2% CMC was made up at each pH in the buffers listed. No more than 10 µl of enzyme was added to the total reaction mixture of 0.5 ml so that the pH of the reaction would not be effected.

#### Thermal Stability of Cellulases

[0119] The thermal stability of these proteins is summarized in Table IV. The addition of CBDs to the catalytic domains

has different effects on the thermal stability of the protein constructs. The CelE1 was dramatically stabilized by the addition of the cellulose binding domains, there is a 25° C increase in the stability of the CelE1/2 relative to CelE1.

[0120] Assays to determine the thermostability of the cellulases with time were carried out in one of two ways depending on the temperature at which the studies were done and the time of incubation. At temperatures of up to 80° C or if the samples were incubated for less than two minutes then stability studies were done by protocol 1. An aliquot (40 µl) of the purified cellulase was diluted into an aliquot (200 µL) of incubation buffer, 50 mM sodium phosphate buffer at pH 7.0, that was preheated in an 80° C water bath. At the specified time points aliquots (25 µl) were withdrawn from the diluted sample incubated at the designated temperature and diluted into 475 µl of ice cold incubation buffer. Each of the time points was then assayed to determine the remaining cellulase activity using the standard CMCase assay.

[0121] Protocol 2 was used when incubations of above 80° C were done for a time in which any assay point exceeded two minutes of incubation time. In this case sufficient cellulase for an individual CMCase assay was placed in a tube and preheated to 80° C. At time 0 the samples were then transferred to a water bath at a higher temperature for example 85° or 90°. At the designated time points the samples were withdrawn and placed in an ice water bath. Each of the time end points was then assayed to determine the remaining cellulase activity with time using the standard CMCase assay.

#### Structural characterization of purified cellulases

[0122] Characterization of the CelB5 protein by MALDI-TOF and N-terminal sequencing shows the linker domain is clipped between T999 and A1000 in the full length CelB protein sequence and that the two C-terminal amino acids K1424 and N1425 are also proteolyzed (Figure 15). The N-terminal sequence of the expressed proteins were determined using the techniques of Matsudaria (1987) in which proteins were electrophoresed on SDS PAGE, blotted to PVDF membranes and then N-terminally sequenced by Edman degradation (Figure 15).

#### Application Testing of Tok7B.1 Cellulase Constructs

[0123] The purified enzymes were tested in the denim stone-wash application, under the same conditions that were used in the initial evaluation of the cellulase supernatants. Results are shown in Table IV. Cellulase constructs that gave a stonewashing effect and showed a dose dependent increase in abrasion with increasing concentrations of enzyme were lacking a cellulose binding domain. Results demonstrated the CelB5 and CelE1 protein constructs gave the best stonewash effect.

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Annex to the description

## SEQUENCE LISTING

## (1) GENERAL INFORMATION

## (i) APPLICANT

(A) NAME: CLARIANT FINANCE (BVI) LIMITED  
 (B) STREET: Citco Building, Wickhams Cay, P.O. Box 662  
 (C) CITY: Road Town  
 (D) STATE OR PROVINCE: Tortola  
 (E) COUNTRY: British Virgin Islands  
 (F) POSTAL CODE:

(ii) TITLE OF THE INVENTION: Truncated Cellulase Compositions

(iii) NUMBER OF SEQUENCES: 47

## (iv) COMPUTER-READABLE FORM:

(A) MEDIUM TYPE: Diskette  
 (B) COMPUTER: IBM Compatible  
 (C) OPERATING SYSTEM: DOS  
 (D) SOFTWARE: FastSEQ for Windows Version 2.0

## (v) CURRENT APPLICATION DATA:

(A) APPLICATION NUMBER: 98810919.5

## (2) INFORMATION FOR SEQ ID NO:1:

## (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 11707 base pairs  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

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TTGTTGATGA	TGTTGCTCG	ATACTTACAT	TCTGGCAGGA	TTCGATATTA	AGCAAAAAAG	240
ATGAGATTAG	AAACATGGTT	GGTGGAGATT	GGAAAAAACC	TCCTGCAGAG	CAGGTTGTTG	300
CTGGACCACC	TGCTGAATAC	AAGTGGTATG	CAACTGCTCA	AATCAATGAC	AGCGATTTT	360
ACAACTCAAA	TCTCATACCT	CCGTTGCAAA	GTGGTGACAG	TCTCGTACTT	ATGACAACAC	420
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CTGAATATAC	AGGATTCAAT	GTCAATAGCA	ACGGTGATCT	AAAAATTATA	TGGGACAGAC	540
CGAGCCAGCA	AACAATAAAT	GAAATTACCA	ATGATTTGAA	TTTGCCAATT	GTTCCAACAC	600
CAACGCCTGT	GCCAACAGCA	AATGTAACCA	CGGGTACCAC	AAACAATTTC	CAAATAATAA	660
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CGCTTGTAGG	TACTTTGATA	TTTCATCAGG	AAGCAAAAGC	AGCAGCATAT	ACTGTTGATT	840
TTGAAGGTGC	TGATACTTTA	TCTTACTTTG	CTTATGGAAA	ATCGAGCATA	GCAGTTGACA	900
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AGAAAATTGT	TGGTAAATGG	ACTCCAAATA	TTAGCAATGT	CAGGAATTTG	TTAATTGTAA	1200
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ATAATAGTTA	CCTATCAAAAT	GCAGTGACAT	TTTCAAGTGG	ATTTGAAAGT	GGCACTACCG	1320
AGGGTTGGCA	GGCAAGGGGA	AGCGGTGTTA	CAGTAAACC	AGATAGCGTT	GTGGCATATA	1380

	GTGGCAAGTA	TAGTTTGTAC	GTCAGTGGAA	GAACGTCAAA	TTGGCATGGT	GCACAGATTC	1440
	CGGTAGATAC	AAATTTTGGAA	CAGGGTAAAG	TGTATAAAAT	AAGTGTTTGG	GTTTATCAGA	1500
	ACAGTGGTTC	AACTCAAAAA	ATGTCATTAA	CTATGCAAAG	AAGATTTGCT	ACAGATCCTT	1560
5	CAACAAGCTA	TGAAAATCTG	ATATATAACA	GGGATGTACC	GAGTAATACG	TGGGTTGAGC	1620
	TGAGTGGAAAG	CTACTCAATT	CCTGCTGGTG	TTACAGTTAG	CGAGTTGTTG	CTTTATGTTG	1680
	AGGCACAAAA	TGCAAATTG	GCTTTCTGGG	TTGATGATTT	AAAGATTTAT	GATTTATCCA	1740
	AGTTGGCTGA	ACCTGAATGG	GAGATACCAT	CTTTGATAGA	AAAGTATAGA	GATTATTCCA	1800
	AAGTAGGAGT	AGCTTTGTCT	TACAAAAGCA	TTGCCTCTGA	TACAGAAAAG	AAGATGGTTT	1860
	TGAAGCATTT	CAATAGTATT	ACTGCAGGGA	ACGAAATGAA	ACCATCAGAG	TTACTTGTCT	1920
10	ATGAAAATAC	TTACAACCTT	AGCAAAGCAG	ACGAATTTGT	AAATTTTGCA	ACAAGTAACA	1980
	ACATTGCCAT	CAGAGGTCAT	ACACTGGTTT	GGCATGAGCA	AACACCCGAC	TGGTTTTTCA	2040
	AGGACACAAA	TGGAATACG	TTGAGCAAGG	ATGCATTGCT	AAGCAGATTA	AAACAGTATA	2100
	TTTATACGGT	AGTGGGAAGA	TATAAAGGGA	AGGTTTATGC	ATGGGATGTG	GTAATGAAG	2160
	CAATAGATGA	AAGTCAAGGT	GATGGATTCA	GGAGATCTAA	CTGGTACAAC	ATTTGTAGTC	2220
	CCGAATATAT	TGAGAAGGCT	TTTATATGGG	CACATGAAGC	CGATCCAGAC	GCAAAATTGT	2280
15	TTTACAACGA	TTACAACACA	GAAAACAGTC	AGAAGAGACA	GTTTATTTAC	AACATGATTA	2340
	AGAGTCTCAA	GGAAAAAGGT	GTTCCAATTC	ATGGAATAGG	ATTGCAGAGT	CATATAAATC	2400
	TTGATTGGCC	CTCGATTAGC	GAGATAGAGA	ACACCATAAG	ATTGTTTCAGC	TCTATACCTG	2460
	GATTGGAGAT	ACACATTACG	GAGCTTGATA	TGAGTTTTTA	TCAGTGGGGT	TCGAGTACCA	2520
	GTTACTCAAC	GCCACCAAGA	GATCTCCTGA	TAAAACAGGC	AATGAGATAT	AAGGAGTTAT	2580
20	TTGATTGTGTT	TAAAAAGTAC	AACAATGTAA	TAACAAGTGT	AACATTCTGG	GGACTGAAGG	2640
	ATGATTACTC	ATGGCTGAGT	CAAAACTTTG	GAAAAAGTGA	TTACCCGTTG	TTATTGTATG	2700
	AAAACATATA	ATCAAAATAT	GCCTTTTGGG	GCCTGATTGA	GCCAACTGTG	ATACCGGCCA	2760
	ACTCAACATT	GCCAGCACCA	CCAGCTATTC	AAATACCTAC	ACCAACTCCC	ACACCAACCC	2820
	CGACACCGAC	AGTGAGTGCA	ACGCCAACAC	CAGCACCGAC	GGCATCACCG	GTAGGTGGCA	2880
	GTTACTGGAC	GCCGAGTGAG	AGTTACAGTG	CGCTGAAGGT	ATGGTATGCG	AATGGGAATT	2940
25	TAAGCAGCCC	GACGAATGTA	TTGAATCCTA	AGATAAAGAT	AGAGAATGTT	GGGACGACAG	3000
	CGGTAGATCT	TACGAGGGTG	AAGSTAAGAT	ACTGGTACAC	GATAGATGGT	GAGGCAACAC	3060
	AGAGTGTAA	TGTAACAAGC	AGCATAGATC	TCGCGTATAT	AGATGTGAAG	TTTGTGAAGC	3120
	TTGGAGCGAA	CGCAGGCGGA	GCGGATTACT	ATGTGGAGAT	AGGCTTTAAG	AGTGGAGCAG	3180
	GGGTTTGGC	AGCAGGGCAA	AGCACGAAGG	AGATAAGACT	TAGCATACAG	AAGGGCAGTG	3240
30	GCAGCTACAA	TCAGTCAAAT	GACTATTCCG	TGAGGAGTGC	AACAGGCTAT	ATAGAGAACG	3300
	AGAAGGTAAC	AGGGTATATA	GATGATGTAC	TTGTATGGGG	AAGAGAGCCG	AGCAGGAACG	3360
	CCCAGATCAA	GGTATGGTAT	GCGAATGGGA	ATTTAAGCAG	CCCAGCAAT	GTATTGAATC	3420
	CTAAGATAAA	GATAGAGAAT	GTTGGGACGA	CAGCGGTAGA	TCTTAGCAGG	GTGAAGGTAA	3480
	GATACTGGTA	CACGATAGAT	GGTGAGGCAA	CACAGAGTGT	AAGTGTAACA	AGCAGCATAA	3540
	ACCCTGCGTA	TATAGATGTG	AAGTTTGTGA	AGCTTGGAGC	AAATGCAGGT	GGAGCGGATT	3600
35	ACTATGTGGA	GATAGGCTTT	AAGAGTGGAG	CAGGGGTTTT	GGCAGCAGGG	CAGAGCACGA	3660
	AGGAGATAAG	ACTTAGCATA	CAGAAGGGCA	GTGGCAGCTA	CAATCAGTCA	AATGACTATT	3720
	CGGTGAGGAG	TGCAACAGGC	TATATAGAGA	ACGAGAAGGT	AACGGGGTAT	ATAGATGGTG	3780
	CGATAGTGTG	GGGAAGAGAG	CCGAGCAGGG	GTACAAAGCC	GGCGGGAGTA	GTAACACCGA	3840
	CACCGGCACC	GACCCCGACA	TCGACGCCGA	CACCAACACC	TACAACCACA	CCTGCACCGA	3900
	CATCAGCCCC	GACACCGAGC	CCAACAGTGA	CAGCAACGCC	GACTCCAACG	CCGACGCCGA	3960
40	CAGTGACGGT	TACTGTGACT	CCGACACCGA	CACCAACACC	GACGCCGACA	CCGACAGGGA	4020
	CACCTGGCAC	GGGAAGTGGT	TTGAAGGTAC	TATACAAGAA	CAATGAGACA	AGTGCGAGCA	4080
	CAAGTTCTAT	AAGGCCGTGG	TTTAAGATAG	TGAATGGAGG	CAGCAGCAGT	GTTGATCTTA	4140
	GCAGGGTTAA	GATAAGATAC	TGGTACACAG	TGGATGGTGA	CAAGCCACAG	AGTGCGGTAT	4200
	GTGACTGGGC	ACAGATAGGG	GCAAGCAATG	TGACATTCAA	TTTTGTGAAG	CTGAGCAGCG	4260
45	GAGTGAGTGG	AGCGGATTAT	TACTTGGAGG	TAGGATTTAG	CAGTGGAGCT	GGGCAGTTGC	4320
	AGCCTGGTAA	GGACACAGGG	GATATACAGG	TAAGGTTTAA	CAAGAATGAC	TGGAGCAATT	4380
	ACAATCAGGC	AGACGACTGG	TCATGGTTGC	AGAGCATGAC	GAATTATGGA	GAGAATGCGA	4440
	AGGTAACGCT	GTATGTAGAT	GGTGTTCCTG	TATGGGGGCA	GGAGCCGGGC	GGAGCGACAC	4500
	CTGCACCGAC	AAGCACAGCA	ACACCAACGC	CAACTCCGAC	AGCAACAGCA	ACACCGACGC	4560
	CGACAGCAAC	GCCAACGTCT	ACACCGACAC	CGACAGCAAC	ACCAACCCCA	ATACCAACAC	4620
50	CCACAACGCC	TCCTACAAAA	CCGGTGGGTA	AGATTCCACC	AAATAACAAC	CCGCTGATTT	4680
	CACACAAGTT	CGGTGCGGAC	CCGGCAGTCC	TTGTTTATGG	TGGCAGAGTT	TATATGTATC	4740
	TTACAAAGTA	CATTCTGGAG	TATGATGAAA	ATGGAATGT	GAAGGATAAC	TCATACAGCA	4800
	AAATAAACAA	AATAACAGTT	ATATCATCGG	ATGACCTTGT	AAACTGGACA	GACCATGGCG	4860
	AGATTGAAGT	TGCAGGTCCG	AACGGGGTTG	CAAAATGGGC	AAGTCTTTCA	TGGGCACCGG	4920
	CTGTTGCATG	CAAAAAGATT	AACGGAAAAG	ACAGGTTCTT	CCTTTACTTT	GGCAACAGCG	4980
55	GTGGTGGCAT	AGGTGTAATA	ACGGCAGACT	CACCAACCGG	TCCGTGGTCA	GACCCGCTTG	5040

	GAAGACCGCT	TATCACATGG	TCAACACCCG	GTGTGCAGGG	TGTTGTCTGG	TTGTTTGACC	5100
	CTGCAGTGCT	GGTGGATGAT	GACGGGAAAG	CATATATTTA	TTTGGGTGGA	GGAGTTCCAC	5160
5	AGGGGCAGGA	TGCTATGCCA	AACACGGCAC	GTGTGATGCA	GCTGGGAGAT	GATATGATAA	5220
	GTGTTGTTGG	GAGTGCTGTT	ACAATTCCAG	CACCATACAT	GTTTGAGGAT	TCCGGGATAA	5280
	ACAAGATAGG	GAATACCTAC	TATTACTCCT	ACTGCACAAA	CTTTGCACAA	AGACCCGACG	5340
	GCAGCCACC	GGCGGGTGCT	ATAGCGTACA	TGACAGGCAG	AAGTCCAATG	GGACCCTGGG	5400
	AATACCGGG	GGTTATACTC	AGAAATCCGG	GGAATTTCTT	TGGAGTTGGT	GGCAATAACC	5460
	ATCACCAGCT	GTTTGAATTT	AATGGCAAAT	GGTATATTGC	ATACCACGCA	CAGACACTTG	5520
10	CAAAAGATTT	GGGAGTTGCA	AAGGGTTACA	GGTCACCGCA	TATAAACTAT	GTGCAGATTG	5580
	AAAATGGTAC	GATAAAAAAA	GTAACAGCCG	ACTACAAAGG	AGTGGCACAG	GTGAAGAATT	5640
	TTGACCCGTA	CAGGATGGTT	GAGGCGGAGA	CATTTGCATG	GTGTGCAGGG	ATTTCCAGAA	5700
	AGAAGGCCAA	TGCGAGCAAT	AATATGTGCT	TGACAGGTAT	AGACAGTGA	GACTGGATTG	5760
	CACTTTCCAA	GGTTGACTTT	GGTAATGCAG	GTCCACAGAA	ATTTGAGGCG	CAGGTTTCCA	5820
	GCATCAACGG	CAAAGGTGAT	ATAGAATCTA	GGATGATGCT	GGTTGATGGT	AGAACCATTG	5880
15	CAGTTGCAGA	GGTCTGCCA	CAGAGTGGTT	CTTCTTCGCA	GTGGGTCAAA	GTAGAGGCAA	5940
	ATGTTGAGAA	TGTAACAGGT	GTGCATGATT	TGTATCTTGT	GTTCAAGAGT	GAAAAGAAGA	6000
	GCAACCTGTT	TGACATGGAT	TGGTGGAGAT	TTGTGAGGTA	AATAGCATT	GTCAACGCGA	6060
	GATATTAATA	CTGCTTAGC	AGTCAGTAAA	TGAATGAATA	AAGGAATTTT	AGCGGGGTAG	6120
	CACATCTATA	GGAAAGATGT	GCTGCTTCGC	TAAAGTCTTA	TATATGGGTG	TTTCAAAAGT	6180
	AGCACAAAAG	ATAATTGGTT	TTAACAGTCA	AAATGTACAA	GTAAAAGTAA	ACAAGCAGGA	6240
20	GGGGAGTTAG	TGAAATGAAA	AAGAGAGTTT	TAAGGTTTGT	TTCCCGTTA	ATATTGGCAG	6300
	TGTTTATTAT	GAGCATAAGT	TTAGTGGGAT	CAATGAGTTA	TTTTCTGTGA	AAGACCGAAG	6360
	CTGCACCTGA	CTGGAGTATA	CCGAGTTTAT	GGGAGAGTTA	TAAGAATGAT	TTTAAGATAG	6420
	GGGTAGCGAT	ACCTGCGAGA	TGTTTGAGCA	ATGATACAGA	CAAGCAAATG	GTGTTGAAGC	6480
	ATTTTAACAG	TATTACAGCA	GAGAACTGAG	TGAAGCCTGA	AAGTTTATTG	GCGGGGCAGA	6540
25	CAAGCACGGG	ATTGAGTTAC	AGGTTTAGCA	CAGCTGATAC	GTTTGTTAAC	TTTGGCAATA	6600
	CGAACAAATAT	AGGGATTAGA	GGGCATACAC	TGGTATGGCA	TAATCAAACA	CCTGATTGGT	6660
	TTTTTAGAGA	CAGCAGTGGG	CAGATGTTAT	CGAAAGATGC	ACTGTTAGCG	AGGCTGAAGC	6720
	AATACATTTA	TGATGTTGTT	GGCAGGTATA	AGGGTAAGGT	ATATGCATGG	GACGTTGTAA	6780
	ATGAGGCTAT	AGATGAGAGT	CAGCCTGATG	GATATAGACG	TTCGACATGG	TATCAAATCT	6840
30	GTGGTCCGGA	GTATATAGAG	AAGGCATTCA	TATGGGCGCA	CGAAGCCGAT	CCGAATGCGA	6900
	AGCTGTTTTA	TAATGACTAT	AATACAGAGA	TTTCAACAAA	GAGAGATTTT	ATATACAACA	6960
	TGGTAAAGAA	TTTAAATCC	AAGGGTGTGC	CGATTTCATG	TATAGGGATG	CAGAGCCATA	7020
	TAAACGTGAA	CTGGCCATCG	GTGAGTGAGA	TAGAGAACAG	TATAAACTG	TTAGTTCTGA	7080
	TACCTGGGAT	TGAGATTAC	ATTACAGAGC	TTGACATGAG	TTTATACAAC	TATGGATCAA	7140
	ACGAGAATTA	TTCAACACCG	CCGCAGGATT	TGCTTCAGAG	GCAGGCACAG	AAGTACAAAG	7200
35	ATATATTTAC	AATGCTGAGG	AAATACAAAG	GTATTGTAAC	ATGTGTTACA	TTCTGGGGTT	7260
	TGAAGGATGA	CTATTCATGG	CTGAACATCA	CCAGTAAGAG	GGATTGGCCG	CTGTTGTTTT	7320
	TTGATGATTA	CATGCAAAAG	CCGGCGTATT	TGCTGGGTAT	TGAGGCAGCA	GGTGCAAGTG	7380
	CATCTCCAAG	CCCGACAGTG	ACAGCAACGC	CGACGCCGAC	TCCGACGCCG	ACAGTGAAGT	7440
	TTACGGCGAC	TCCGACACCG	ACACCAACAG	GGACACCTGG	TACGGGAAGT	GGTTTGAAGG	7500
	TACTATACAA	GAACAATGAG	ACCAAGTGC	GCACAGGTTT	TATAAGGCCG	TGGTTTAAGA	7560
40	TAGTGAATGG	AGGCAGCAGC	AGTGTGTGAT	TTAGCAGGGT	TAAGATAAGA	TACTGGTACA	7620
	CAGTGGATGG	TGACAAGCCA	CAGAGTGCGG	TATGTGACTG	GGCACAGATA	GGTGCAAGCA	7680
	ATGTGACATT	CAATTTTGTG	AAGCTGAGCA	GCGGAGTGAG	TGGAGCGGAT	TATTACTTGG	7740
	AGGTAGGATT	TAGCAGTGG	GCTGGGCAGT	TGCAGCCTGG	TAAGGACGCA	GGGGATATAC	7800
	AGGTAAGGTT	TAACAAGAAT	GACTGGAGCA	ATTACAATCA	GGCAGACGAC	TGGTCATGGT	7860
	TGCAGAGCAT	GACGGATTAT	GGAGAGAATG	CGAAGGTGAC	GCTGTATGTA	GATGGTGTTT	7920
45	TGGTATGGGG	GCAGGAGCCG	GGAGGAGCGA	CACCTGCACC	GACAGCGACA	GCAACACCAA	7980
	CGCCAAATTC	GACAGCAACA	GTAACACCGA	CGCCGACAGC	AACTCCAACG	TCTACACCGA	8040
	GACCGACAGC	GACAGCGACC	CCGACACCGA	CAGTGAGTGC	AACGCCAACA	CCGGCACCAG	8100
	CGGCATCACC	GGTAGGTGGC	AGTTACTGGA	CGCCGAGTGA	GAGTTACGGT	GCGCTGAAGG	8160
	TATGGTATGC	GAATGGGAAT	TTAAGCAGCC	CGACGAATGT	ATTGAATCCT	AAGATAAAGA	8220
	TAGAGAATGT	TGGGACGACA	CCGGTAGATC	TTAGCAGGGT	GAAGGTAAGA	TACTGGTACA	8280
50	CGATAGATGG	TGAGGCAACA	CAGAGTGTA	GTGTAGCGAG	CAGCATAAAT	CCTGCGTATA	8340
	TAGATGTGAA	GCTTGGAGCG	AACGCAGGCG	GAGCGGATTA	CTATGTAGAG	ATAGGGTTTTA	8400
	AGAGTGGAGC	AGGTGTTTTG	GCAGCAGGGC	AGAGCACGAA	GGAGATAAGA	CTTAGCATAC	8460
	AGAAGGGCAG	TGGCAGCTAC	AATCAGTCAA	ATGACTATTC	GGTGAGGAGT	GCAACAGGCT	8520
	ATATAGAGAA	CGAGAAGGTA	ACGGGGTATA	TAGATGATGT	ACTTGATATG	GGGAGAGAGC	8580
55	CGAGCAGGAA	CGCCAGATC	AAGGTATGGT	ATGCGAATGG	GAATTTAAGC	AGCCCGACGA	8640
	ATGTATTGAA	TCCTAAGATA	AAAATAGAGA	ATGTTGGGAC	GACAGCGGTA	GATCTTAGCA	8700



	GGGTGAAGGT	AAGATACTGG	TACACGATAG	ATGGTGAGGC	AACACAGAGT	GTAAGTGTA	8760
	CAAGCAGCAT	AAATCCTGCG	TATATAGATG	TGAAGTTTGT	GAAGCTTGGA	GCAAATGCAG	8820
	GCGGAGCGGA	TTACTATGTA	GAGATAGGGT	TTAAGAGTGG	AGCAGGTGTT	TTGGCAGCAG	8880
5	GGCAGAGCAC	GAAGGAGATA	AGGCTTAGCA	TACAGAAGGG	CAGTGGCAGC	TACAATCAGT	8940
	CAAATGACTA	TTCGATAAGA	AGTGCGAATA	GCTATATAGA	GAACGAGAAG	GTAACAGGGT	9000
	ATATAGATGG	TGCGATAGTG	TGGGGAAGAG	AGCCGAGCAG	GGGTACAAAG	CCGGCGGGAG	9060
	TAGTAACACC	GACACCGGCA	CCGACCCCGA	CATCGACGCC	AACACCGATA	CCTACAACCA	9120
	CACCGACACC	GACACCGACA	CCGACTGTGA	CGGTGACCCC	AACTTCTACA	CCCACACCGG	9180
	TTTCATCATC	CACTCCTACA	CCAACAGCAA	CGCCAACACC	TACACCTTCT	ATCACGATAA	9240
10	CACCAGCGCC	AACTGCAACA	CCCCTCCGA	CTCCTTCTGT	CACAGATGAT	ACAAATGATG	9300
	ATTGGTTATT	TGCGCAGGGT	AACAAAATAG	TCGACAAGGA	TGGCAAACCT	GTATGGTTAA	9360
	CAGGAGTTAA	TTGGTTTGGA	TTTAATACAG	GAACGAATGT	GTTTGATGGT	GTGTGGAGTT	9420
	GTAATCTTAA	AAGTGCATTA	GCTGAGATTG	CAAACAGAGG	ATTTAATTTG	CTAAGAGTAC	9480
	CGATTTTCAGC	AGAGCTGATT	TTGAATTGGT	CGAAAGGAAT	TTATCCAAAA	CCAAATATCA	9540
15	ATTATTATGT	TAACCCTGAG	TTAGAAGGTC	TGACGAGTTT	AGAGGTATTT	GATTTTGTAG	9600
	TAAAAACATG	CAAAGAAGTT	GGACTGAAAA	TTATGTTGGA	TATTCATAGT	GCAAAAACCTG	9660
	ATGCGATGGG	GCATATATAT	CCGGTATGGT	ATACAGATAC	TATAACGCCA	GAAGATTATT	9720
	ATAAAGCATG	TGAATGGATC	ACAGAGAGAT	ATAAAAAATGA	TGATACAATT	GTAGCATTTG	9780
	ATTTGAAGAA	TGAGCCACAT	GGTAAACCAT	GGCAAGATAG	TGTTTTTGCA	AAATGGGACA	9840
	ATTCAACAGA	TATTAACAAC	TGGAAATATG	CTGTGAGAC	CTGTGCGAAG	AGAATAGTTG	9900
20	CAAAAAATCC	AAACATGTTA	ATAGTAATTG	AAGGAATAGA	AGCTTATCCA	AAAGATGATG	9960
	TTACGTGGAC	TTCTAAATCA	TCAAGTGACT	ATTATCTAC	CTGGTGGGGC	GGCAACTTAC	10020
	GGGGTGTTAA	AAAGTATCCA	ATAAACCTTG	GACAGTATCA	GAACAAAGTG	GTTTATTTCAC	10080
	CACATGATTA	TGGACCATTG	GTTTACCAGC	AACCCTGGTT	TTATCCTGGA	TTTACCAAAG	10140
	ATACGCTTTA	CAATGATTGC	TGGAGGGATA	ATTGGACTTA	TATTATGGAT	AATGGGATAG	10200
25	CTCCGTTGCT	CATTGGTGAA	TGGGGTGGTT	ACTTAGATGG	TGGCGATAAT	GAAAAGTGGA	10260
	TGACTTATTT	GAGAGATTAT	ATTATAGAAA	ACCATATTCA	TCATACATTC	TGGTGTTACA	10320
	ATGCAAAATC	TGGTGATACT	GGAGGATTGG	TGGGATATGA	TTTTTCGACG	TGGGATGAAC	10380
	AGAAGTACAA	TTTCTTAAAA	CCAGCTTTAT	GGCAGGATAG	TAAAGGAAGA	TTTGTGGGGC	10440
	TTGATCACAA	GAGACCACTG	GGTACAAATG	GGAAGAATAT	AAATATAACT	ATTTATTACC	10500
	AGAACGGTGA	AAAACCGCCT	GTCCCAAAGA	ATTAATAAAT	GGATGAATAC	TTCTTTTGTA	10560
30	AATGTGATGG	AGGCTACTCA	AAGTTGATTT	GGTAGCCTCT	ATCTTTTAA	AAAAGTGGCT	10620
	CTATAATAAA	TATTATTGTG	GGGAGAAAGG	GAAAAATATA	GCACCTATTTC	TGAATGCCTG	10680
	CTAAATAGAA	ACTGTTTTAT	GACAAAAGAA	CACTCAAAAA	GAAAGGAGGC	ATCCAGAATA	10740
	AGTGCTTAAA	TCTAATTATA	TCACTGGACT	TTTAAATTCG	AAAGATATCA	TTCTTCTTCA	10800
	AATGGATGAG	AATGAAAGTG	AAATAGAACT	TCACATAAGT	TACAAGCAAG	GTACATGATT	10860
35	ATCACACGCA	GAAGGTAAAA	GACATACCTA	GACATACCTA	TAATAATGGG	CAAGAAAACA	10920
	ATTTGATTAT	AAGAAAGAGA	AGATATGTCT	GCAAAGCATG	TGGGAAGAAG	TTTTTTGAAC	10980
	ACATAAGTTT	TATAGGCAAA	TCTCAAAGGA	TGACAAATAG	ACTTGCAGCA	TATATTATAA	11040
	GTCAACTTGG	AAGTTTAAAC	AGTAGGAAAG	AGTAGCAAAA	ACACACAAAT	GTTTCAGATG	11100
	TAGCAGTTAT	GAGATTGTTT	GATAAAGTAA	ACCCTGGTCA	AACTCTAGAT	GAGTTTTCTT	11160
	CTGAAGCAAT	ATGGGCAGAA	TAGTTTAAAG	GCAATGCTTT	TGCAAAATTC	TGATGCACTT	11220
40	AAAAAATGA	CAGATTCCAT	GCAAAGTTC	AAAGATTATT	TTGAAGGATA	CAAAAACACA	11280
	AATGTATTCT	ATGTGCCACC	CGGGGCTTTT	GAAAGCAGTA	ATTTTGAAAA	AGCACTGGAT	11340
	TCATACATGG	ATAAGAGCAG	AAAAATAACA	AAAATTATCC	TTATCCTTGA	CACCAATCCT	11400
	TATACAAACA	AAGCAATAGA	TATTGTTGAC	AGTGTTGAAA	AGGTATTGAA	AAACTCTTTG	11460
	GAGTTTGTTG	ATACAAAATT	CACTGAATTT	GGTGTTGGCG	GAATATCGTC	AAGCAATCAC	11520
45	GATTTGAGAA	GCATCTATTT	TAAAGATTTT	AGGACTTTGA	GACTCATAAT	GATAATTAGC	11580
	ATTTTGCTTT	TGATGTTTCA	AATTTCAAGA	TCAATATTCA	ATGCGGCAGC	GGTTGTGGCA	11640
	ATAGTTTTTA	TTGATTACTA	CCTTGCACTT	TCTATTACAG	AGATGATTTT	CAAAGGTATT	11700
	TTCAATT						11707

## (2) INFORMATION FOR SEQ ID NO:2:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 6416 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

EP 0 921 188 A2

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

	GTCGACACTT	GACTGRRGCG	GGCAGCCGGA	TACATGGAAT	GGGACATATA	CGGGCAATCC	60
5	AAATCTGCAT	GTGAAGATAG	TGGATTATGG	AACAGATTTG	GGTATAACTG	CATCACTTGC	120
	GAATGCGCTT	TTGTACTACA	GTGCGGCGAC	GAANGAGTAT	GGAGTATCTG	ATGAGGCAGC	180
	GAANAATTTA	GCGAAAGAGC	TGCTGGACAG	GATGTGGAAC	TTATACAGGG	ATGACAAGGG	240
	CTTGTCGGCA	CCCGAGAAGA	GAGGAGATTA	CAAGAGSTTC	TTTGAGCAAG	AGGTATACAT	300
	TCCAGCGGGC	TGGACAGGGA	AGATGCCGAA	TGGATGTGTA	ATAAAGAGTG	GAGTGAAGTT	360
	TATAGACATA	AGGAGCAAGT	ACAAACAGGA	TGCTGACTGG	CAGAAGCTGG	TTTCGGCATA	420
10	CAATGCAGGA	GAGGCACCGG	AGTTCAGGTA	TCACAGATTC	TGGGCACAGT	GTGATATAGC	480
	AATTGCCAAT	GCAACATATG	AAATCCTGTT	CGGCAATCAG	TAAGTCAAAA	GTGGGTGTGT	540
	GAAAGATATT	AGGAAGGGAA	GTAGCACCGC	TCTGTGCTAC	TTCCCCAATT	TGAAAAGTTA	600
	AATAAAAACA	AGTTAATTA	AGAGAGGGGT	AGGATGCAAG	AAATGAAAGC	AATTAAGAGG	660
	GTTGTCTCGA	TAACTGCTCT	ACTTGTTTTG	ACACTTTCAT	TATGTTTTCC	TGTTATCATG	720
	CCTGTGAAAG	CTTATGCAGG	GGGAACATAT	AATTACGGTG	AGGCACTACA	GAAAACAATA	780
15	ATGTTCTATG	AATTCCAGAT	GTCAGGGAAA	CTACCTTCCT	GGGTAAGGAA	CAACTGGAGA	840
	GGTGACTCTG	GCTTAGATGA	TGGCAAGGAT	GTAGGGCTTG	ATTTAACAGG	TGGCTGGCAT	900
	GACGCAGGTG	ATCACGTAAA	GTTTAACCTG	CCAATGTCGT	ATAGCGCCTC	AATGCTGGGG	960
	TGGGCTGTTT	ATGAATATAA	GGATGCATTT	GTAAAGAGCA	AACAATTGGA	GCACATTTTA	1020
	AATCAAATAG	AGTGGGCAAA	TGACTACTTT	GTGAAGTGTC	ATCCATCAAA	ATATGTATAC	1080
20	TATTATCAGG	TTGGTGATCC	AACTGTAGAT	CACAATTTTT	GGGGACCTGC	AGAAGTAATG	1140
	CAAATGAAAC	GTCCAGCGTA	TAAGTGTGAT	TTATCAAACC	CAGCATCTTC	TGTAGTGGCA	1200
	GAAACAGCTG	CATCACTTGC	GGTGGCTTCA	GTTGTAATAA	AGGAAAGAAA	TTCTCAGAAA	1260
	GCAGCTTCTT	ATCTCCAACA	TGCCAAAGAC	CTGTTTGAAT	TTGCCGATAC	CACAAGAAGT	1320
	GATGCGGGGT	ATACTGCTGC	AACAGGTTTC	TACACATCGG	GTGGTTTTAT	TGATGACCTT	1380
	GGATGGGCTG	CTGTATGGCT	TTATATTGCG	ACAAATGACA	GTAGTTATTT	GACGAAAGCT	1440
25	GAAGAGTTGA	TGTCAGAATA	TGCTAATGGT	ACTAATACAT	GGACACAATG	CTGGGATGAT	1500
	GTTTCGATATG	GAACATTGAT	CATGCTTGCA	AAGATTACAG	GGAAAGAGTT	ATATAAAGGA	1560
	GCTGTGGAAG	GAAACTTAGA	CCATTGGACT	GACAGAATTA	CGTATACGCC	GAAAGGGATG	1620
	GCATATCTGA	CAGGATGGGG	TTCAATTAAGA	TATGCGACAA	CAGCTGCATT	TTAGCATGT	1680
	GTCTATGCAG	ACTGGTCAGG	GTGCGATTTC	AACAAAAAGA	CCAAATATTT	GAACCTTGCA	1740
30	AAAAGCCAGA	TTGACTATGC	ACTGGGTTC	ACAGGTAGAA	GTTTTGTAGT	AGGATTTGGC	1800
	ACCAATTATC	CACACATCC	GCATCACAGG	AATGCGCATA	GTTTCATGGG	TAACAGCATG	1860
	AAAATACCAG	AGTATCACAG	ACACATATTA	TATGGAGCAC	TGGTTGGTGG	TCCTGGTAGT	1920
	GATGATAGTT	ATAATGATGA	CATTACCGAT	TATGTACAAA	ATGAGGTTGC	CTGCGATTAT	1980
	AATGCTGGAA	TTGTTGGTGC	ACTGGCAAAG	ATGTACCAGT	TATATGGAGG	TGAACCTATT	2040
	GATGATTTTA	AAGCAATTGA	AACACCCACA	AATGATGAAA	TTTTTGTTGA	ATCAAAATTT	2100
35	GGGAATTCAC	AGGGTCCAAA	TTATACCGAA	GTAATTTCCCT	ATATCTATAA	TCGAACAGGA	2160
	TGGCCACCAA	GGGTAAGTGA	TAAACTAAGT	TTTAAATATT	TTATAGACCT	AACCGAATTA	2220
	ATCCAGGACG	GGTATTCGCC	TGATGTTGTC	AAAGTTGACA	CATACTACAT	CGAAGGAGGT	2280
	AAAATTAGCG	GTCCCTACGT	ATGGGACAAA	AAATGGAAAT	TATACTATGT	TCTGTGGAGT	2340
	TTTAGTGGAA	CCAAGATATA	TCCTGGCGGT	GAAAGTTGAAC	ACAAAAAGCA	GGCTCAATTT	2400
	AAAATATCTG	TTCCGCAGGG	GTATCCATGG	GATCCTACCA	ATGATCCTTC	ATATAAGGGA	2460
40	TTAACCAGTC	AATTAGAAAA	GAATAAATAT	ATTGCCGCAT	ATGATAATAA	TAATCTGGTA	2520
	TGGGGTTTAT	AGCCGGGTGC	GGCAACATCC	ACACCTGCAC	CAACATCAAC	ACCAACACCA	2580
	ACCCCGACCC	CAACACCAAC	AGTGACAGCA	ACGCCGACGC	CGACTCCTAC	ACCGACACCG	2640
	ACGGGGTCAC	CTGGTACGGG	AAGTGGTGTG	AAGGTACTGT	ACAAGAACAA	TGAGACAAGT	2700
	GCGAGCACAG	GTTCTATAAG	GCCGTGGTTT	AAGATAGTGA	ATGGAGGCAG	CAGCAGTGTT	2760
	GATCTTAGCA	GGGTAAAGAT	AAGATACTGG	TACACAGTGG	ATGGTGACAA	GCCACAGAGT	2820
45	GCGGTATGTG	ACTGGGCACA	GATAGGGGCA	AGCAATGTGA	CATTCAATTT	TGTGAAGCTT	2880
	AGCAGCGGAG	TGAGTGGAGC	GGATTATTAC	CTGGAGGTAG	GATTTAGCAG	TGGAGCTGGG	2940
	CAGTTGCAGC	CTGGTAAGGA	CACAGGGGAT	ATACAGGTAA	GGTTTAACAA	GAATGACTGG	3000
	AGCAATTACA	ATCAGGCAGA	CGACTGGTCA	TGGTTGCAGA	GCATGACGAA	TTATGGAGAG	3060
	AATGCGAAGG	TGACGCTGTA	TGTAGATGGT	GTTCTGGTAT	GGGGGCAGGA	GCCGGGAGGA	3120
50	GCGACACCTG	CACCGACAAG	CACAGCAACA	CCAAGCCCAA	CTCCGACAGC	AACCCCAACA	3180
	CCTACACCTA	CACCGACCCC	GACACCGACA	GAGAGTGCAA	CGCCAAACACC	GGCACCAGCG	3240
	GCATCACCGG	TAGGTGGCAG	TTACTGGACG	CCGAGTGAGA	GTTACGGTGC	GCTGAAGGTA	3300
	TGGTATGCGA	ATGGGAATTT	AAGCAGCCCG	ACGAATGTAT	TGAATCCTAA	GATAAAGATA	3360
	GAGAATGTTG	GGACGACAGC	GGTAGATCTT	AGCAGGGTGA	AGGTAAGATA	CTGGTACACG	3420
	ATAGATGGTG	AGGCGACACA	GAGTGTAAGT	GTAGCGAGCA	GCATAAATCC	TGCGTATATA	3480
55	GATGTGAAGT	TTGTGAAGCT	TGGAGCGAAC	GCAGGCGGAG	CGGATTACTA	TGTGGAGATA	3540

	GGCTTTAAGA	GTGGAGCAGG	TGTTTTGGCA	GCAGGGCAGA	GCACGAAGGA	GATAAGGCTT	3600
	AGCATACAGA	AGGGCAGTGG	CAGCTACAAT	CAGTCAAATG	ACTATTCCGT	AAGGAGTGGC	3660
	AATAGCTATA	TAGAGAACGA	GAAGGTAACA	GGGTATATAG	ATGATGTACT	TGTATGGGGA	3720
5	AGAGAGCCGG	GCAGGAACGC	CCAGATCAAG	GTATGGTATG	CGAATGGGAA	TTTAGGCAGC	3780
	ATGACGAATG	TATTGAATCC	TAAGATAAAG	ATAGAGAATG	TTGGGACGAC	AGCGGTAGAT	3840
	CTTAGCAGGG	TGAAGGTAAG	ATACTGGTAC	ACGATAGATG	GTGAGGCGAC	ACAGAGTGTA	3900
	AGTGTAAACA	GCAGCATAAA	TCCTGCGTAT	ATAGATGTGA	AGTTTGTGAA	GCTTGGAGCA	3960
	AATGCAGGTG	GAGCGGATTA	CTATGTGGAG	ATAGGGTTTA	AGAGTGGAGC	AGGTGTTTTG	4020
10	GCAGCAGGGC	AGAGCACGAA	GGAGATAAGG	CTTAGCATAC	AGAAGGGCAG	TGGCAGCTAC	4080
	AATCAGTCAA	ATGACTATTC	GGTAAGAAAT	GCGACAGGCT	ATATAGAGAA	CGAGAAGGTA	4140
	ACAGGATGTA	TAGATGGTGC	GATAGTGTGG	GGAAGAGAGC	CGAGCAGGGG	TACAAAGCCG	4200
	GCGGGAGGAG	TGACACCGAC	ACCGGCACCG	ACGCCGACAT	CGACGCCAAC	ACCAACACCT	4260
	ACAACCACAC	CGACACCGAC	ACCGACTGTG	ACGGTGACCC	CAACTCCTAC	ACCTGCCGTA	4320
	ACCCCCGATG	TTAAATATC	GATCGATACG	TCCAGGGGAA	GAACAAAGAT	AAGCCCGTAT	4380
15	ATTTATGGAG	CAAATCAGGA	TATCCAGGGT	GTTGTTCCAC	CTGCAAGACG	ACTTGGTGGG	4440
	AACAGATTGA	CGGGTTACAA	TTGGGAGAAC	AATATGTCCA	ATGCAGGGAG	TGACTGGTAT	4500
	CATTCAAGCG	ATGATTATAT	GTGTTATATT	ATGGGTATAA	CAGGGAATGA	TAAGAACGTT	4560
	CCAGCAGCTG	TTGTAAGCAA	ATTTACAGAG	CAGTCAATAA	AGCAAATGTC	ATATTCAGCC	4620
	ATCACATTAC	AGATGGTAGG	TTATGTGGCA	AAGGATGGGA	ATGGTACAGT	GAGCGAGTCA	4680
	GAGACAGCTC	CGTCGCCGAG	ATGGGCTGAG	GTCAGTTTAA	AAAAAGATGG	TGCACGTGTA	4740
20	TTGCAGCCTG	ACGTGAATGA	TAACTATGTA	TATATGGATG	AGTTTATTAA	CTATCTGATT	4800
	AATAAGTATG	GTCGATCATC	GTCTGCAACG	GGAATTAAAG	GATATATACT	TGACAACGAG	4860
	CCGGACTTAT	GGTTTACTAC	TCATCCGCGA	ATTCTCCAC	AGAAGGTAAC	CTGCAGTGAA	4920
	TTGATAAATA	AATCGGTGGA	GCTGGCGAAA	GTAAATAAGA	CACTTGATCC	AGATGCAGAA	4980
	ATTTTTGGAC	CTGCATCGTA	TGGTTTTGTG	GGATATTTAA	CATTGCAGGA	TGCACCTGAC	5040
25	TGGAATCAGG	TTAAAGGAAA	TCACAGATGG	TTTTTGAGCT	GGTACCTTGA	GCAGATGAAG	5100
	AAAGCATCGG	ATAGTTTTGG	GAARAGGTTA	TTGGATGTAC	TTGACATACA	CTGGTACCCG	5160
	GAGGCGCAGG	TTGGCGGTGT	GCGAATATGC	TTTGACGGTG	AAAATAGTAC	TTCAAGGGAT	5220
	GTGGCAATAG	CGAGGATGCA	GGCACCAGAG	ACGCTATGGG	ATCCGACATA	TAAACCACC	5280
	CAGAAAGGTC	AGATAACAGC	GGGAGAAAAA	AGCTGATAA	ACCAATGGTT	TCCAGAGTAT	5340
	CTTCCACTGC	TTCCCAATAT	AAAGGCAGAT	ATAGACAAGT	ATTATCCTGG	TACCAAACTT	5400
30	GCTATAACTG	AGTTTGATTA	TGGAGGGAAG	GACCATATAT	CGGGAGGAAT	AGCTTTAGCA	5460
	GATGTGTTAG	GGATATTCGG	CAAGTATGGA	GTATACATGG	CAGCAAGATG	GGGAGATTCC	5520
	GGGAGCTATG	CACAGGCGGC	GTACAACATT	TATCTCAACT	ATGATGGGAA	AGGTTTCGAG	5580
	TACGGTTCAA	CGTGTGTGAG	CGCTGAGACA	ACTGACGTTG	AGAACATGCC	GGTATATGCT	5640
	TCAATTGAGG	GAGAAGATGA	TTGACTGTG	CATATTATAT	TAATTAACAG	GAATTATGAC	5700
	AGGAACTGA	AGGCAGAGAT	AAAGATGAAT	AATACCAGGG	TATACACAGG	TGGAGAGATA	5760
35	TACGGATTG	ACAGTACAAG	CTCTCAGATC	AGGAAGATGG	GAGTGCTCAG	TAATATACAA	5820
	AACAACACAA	TCACCATAGA	AGTTCCAAAT	CTGACGGTAT	ACCATATTGT	TTTAACTTCT	5880
	TCAAAGTAGA	TTAAAGAATA	AAAATGGAGA	CACCTGCTGCA	TGGTAAAAGT	TGAGATGTGC	5940
	AGCAGTGTCT	CATAATCACT	AATCTAATAC	AGTTAGAGAT	GTTAAATTAT	AAAACAGACG	6000
	ATAACTTTGT	TTTAAATGAT	TGNNAGTCGG	ANTTCTNNTG	ATTAAAACAT	NAGAAANTTG	6060
40	TNATANTNGA	CTTTAATTNT	NGCANNATAAA	CGTAAATGGA	TTCAATNACN	WTACRATTNN	6120
	CRTAATCTAW	AAGRAGCACA	GAGAAATATT	ACATAGGAGG	ATGTATCAAT	AAATGATAGA	6180
	TAAAAAGATA	ATTGCTGTTA	CAATTTTART	AATGGTAACA	TACTTTTTAG	TACAAATATC	6240
	RACTATAGGT	GCACGGAATA	TACCAGAGAC	ATANTGGATA	CCGCTGGATA	TAGATACAAT	6300
	AAGTATTGAC	CTGGGCWAGN	AGCCATATGT	GANAGAATTT	ATAGTATATT	TTGGATATGG	6360
	CGGAGGCCAA	ATAGASTGTC	WGTTTTATAG	AGACAATACT	TTGGCATTMT	ACATCA	6416

## (2) INFORMATION FOR SEQ ID NO:3:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 28 base pairs  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

CCTTTATGAA TTCATTTACT GACTGCTA

28

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 31 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

CTTCCTCGA GAATTCACAC ACCCACTTTT G

31

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 30 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

TACCCCTCGA GAATTCCTAT TTACTCATTA

30

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 28 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

CTACACCCAT GGTAACCCCC GATGTAA

28

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 27 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

AAATGCTCGA GTAAAAGTGA ACAAGCA

27

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 30 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

ATGTGTCCAT GGCATTAATT ATTTTGTGTTG

30

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 31 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

ATGCAAGGCA TGCAAGCAAT TAAGAGGGTT G

31

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 32 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

TCAACAAAGA TCTAATCATT TGTGGGTGTT TC

32

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 34 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

GTGCAGCTCG AGCTCCTCCC GGCTCCTGCC CCCA

34

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 39 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

GAGGAACGGT CATATGAAGG TATGGTATGC GAATGGCAA

39

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 38 base pairs

- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

5

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

GAGGAGGAGC ATGCAGATCA AGGTATGGTA TGCGAATG

38

10

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 25 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

15

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

TTTAGCATGC TGAGGAAATA CAAAG

25

20

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 31 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

25

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

AGTTAGTGGC ATGCAAAGA GAGTTTTAAG G

31

30

(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 31 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

35

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

GAAGTATGGA TCCATTTATT AATTCTTTGG G

31

40

(2) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 32 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

45

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

TACAATTTTA GCCATGGTAA CATACTTTTT AG

32

50

55

(2) INFORMATION FOR SEQ ID NO:18:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 34 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

GCAGCAGTGT CGACATTTTT ATTCTTTAAT CTAC

34

(2) INFORMATION FOR SEQ ID NO:19:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 32 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

GTGGATGAGA TCTAACCCGG CTCTAAACCC CA

32

(2) INFORMATION FOR SEQ ID NO:20:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 35 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

TTGAACTTCC CCATGGCAGA ATTTTACAA ATTGG

35

(2) INFORMATION FOR SEQ ID NO:21:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 18 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

TGTATCCCAT GCCGTCTT

18

(2) INFORMATION FOR SEQ ID NO:22:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 26 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear



(xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

CAAAAAGCAA TTATGTTTGA TGAATT

26

(2) INFORMATION FOR SEQ ID NO:23:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 24 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

TGGTGCTGGC AATGTTGAGT TGGC

24

(2) INFORMATION FOR SEQ ID NO:24:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 24 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:24:

TCGGTAGTGC CACTTTCAAA TCCA

24

(2) INFORMATION FOR SEQ ID NO:25:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 36 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:25:

CAAAGCAGAC GAATCTGTGC GTGGTATGCA ATATAC

36

(2) INFORMATION FOR SEQ ID NO:26:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 18 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:26:

AGCTGAGCAG CGGAGTGA

18

(2) INFORMATION FOR SEQ ID NO:27:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 18 base pairs
- (B) TYPE: nucleic acid

(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

5 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:27:

TCCACTCACT CCGCTGCT 18

(2) INFORMATION FOR SEQ ID NO:28:

10 (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 18 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
15 (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:28:

GTTCTGATAC TGTCCAAG 18

20 (2) INFORMATION FOR SEQ ID NO:29:

(i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 18 base pairs  
(B) TYPE: nucleic acid  
25 (C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:29:

30 ACAGGCGGCG TACAACAT 18

(2) INFORMATION FOR SEQ ID NO:30:

(i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 18 base pairs  
35 (B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

40 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:30:

TTGAGGGATA TGGTGACC 18

(2) INFORMATION FOR SEQ ID NO:31:

45 (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 18 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
50 (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:31:

GAGAAACATA TCCTGCAA 18

55 (2) INFORMATION FOR SEQ ID NO:32:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 18 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:32:

CCCATTTTAT ACCCAGGC

18

(2) INFORMATION FOR SEQ ID NO:33:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 18 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:33:

TCTTGAGCAG CCATTGGA

18

(2) INFORMATION FOR SEQ ID NO:34:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 24 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:34:

GATGGCCAGT TCACGTTTAT ATGG

24

(2) INFORMATION FOR SEQ ID NO:35:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 24 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:35:

AGCACTGGTT GGTGGTCCTG GTAG

24

(2) INFORMATION FOR SEQ ID NO:36:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 26 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:36:

GATTGACGGG TTACAATTGG GAGAAC

26

5 (2) INFORMATION FOR SEQ ID NO:37:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 29 base pairs

(B) TYPE: nucleic acid

10 (C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:37:

15 AGWGCACCNA CAAATCCGGC ATTGTARTC

29

(2) INFORMATION FOR SEQ ID NO:38:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 27 base pairs

20 (B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:38:

25 CTCCAGAATG TCATTGTAA GATACAT

27

(2) INFORMATION FOR SEQ ID NO:39:

(i) SEQUENCE CHARACTERISTICS:

30 (A) LENGTH: 35 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other

35

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:39:

GGGAATTCCA TATGGCGGCG TATAATTACG GTGAG

35

(2) INFORMATION FOR SEQ ID NO:40:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 19 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

45 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:40:

50 TATTATTATC ATATGCGGC

19

(2) INFORMATION FOR SEQ ID NO:41:

(i) SEQUENCE CHARACTERISTICS:

55 (A) LENGTH: 18 base pairs

(B) TYPE: nucleic acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:41:

CCAGAGTATC ACAGACAC

(2) INFORMATION FOR SEQ ID NO:42:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 27 base pairs  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:42:

CCTGGATCCC TACGCTCCTC CCGGCTC

(2) INFORMATION FOR SEQ ID NO:43:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 1426 amino acids  
 (B) TYPE: amino acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: None

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:43:

Met	Lys	Lys	Arg	Val	Leu	Arg	Phe	Val	Ser	Arg	Leu	Ile	Leu	Ala	Val
1				5					10					15	
Phe	Ile	Met	Ser	Ile	Ser	Leu	Val	Gly	Ser	Met	Ser	Tyr	Phe	Pro	Val
			20					25					30		
Lys	Thr	Glu	Ala	Ala	Pro	Asp	Trp	Ser	Ile	Pro	Ser	Leu	Trp	Glu	Ser
		35					40					45			
Tyr	Lys	Asn	Asp	Phe	Lys	Ile	Gly	Val	Ala	Ile	Pro	Ala	Arg	Cys	Leu
	50					55					60				
Ser	Asn	Asp	Thr	Asp	Lys	Gln	Met	Val	Leu	Lys	His	Phe	Asn	Ser	Ile
65					70					75				80	
Thr	Ala	Glu	Asn	Glu	Met	Lys	Pro	Glu	Ser	Leu	Leu	Ala	Gly	Gln	Thr
			85						90					95	
Ser	Thr	Gly	Leu	Ser	Tyr	Arg	Phe	Ser	Thr	Ala	Asp	Thr	Phe	Val	Asn
			100					105					110		
Phe	Ala	Asn	Thr	Asn	Asn	Ile	Gly	Ile	Arg	Gly	His	Thr	Leu	Val	Trp
		115					120					125			
His	Asn	Gln	Thr	Pro	Asp	Trp	Phe	Phe	Arg	Asp	Ser	Ser	Gly	Gln	Met
	130					135					140				
Leu	Ser	Lys	Asp	Ala	Leu	Leu	Ala	Arg	Leu	Lys	Gln	Tyr	Ile	Tyr	Asp
145					150					155				160	
Val	Val	Gly	Arg	Tyr	Lys	Gly	Lys	Val	Tyr	Ala	Trp	Asp	Val	Val	Asn
			165						170				175		
Glu	Ala	Ile	Asp	Glu	Ser	Gln	Pro	Asp	Gly	Tyr	Arg	Arg	Ser	Thr	Trp
		180						185					190		
Tyr	Gln	Ile	Cys	Gly	Pro	Glu	Tyr	Ile	Glu	Lys	Ala	Phe	Ile	Trp	Ala

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		195				200				205				
		His Glu Ala Asp Pro Asn Ala Lys Leu Phe Tyr Asn Asp Tyr Asn Thr												
		210				215				220				
5		Glu Ile Ser Thr Lys Arg Asp Phe Ile Tyr Asn Met Val Lys Asn Leu												
		225			230				235					240
		Lys Ser Lys Gly Val Pro Ile His Gly Ile Gly Met Gln Ser His Ile							250				255	
				245										
		Asn Val Asn Trp Pro Ser Val Ser Glu Ile Glu Asn Ser Ile Lys Leu												
				260				265					270	
10		Phe Ser Ser Ile Pro Gly Ile Glu Ile His Ile Thr Glu Leu Asp Met												
				275				280					285	
		Ser Leu Tyr Asn Tyr Gly Ser Asn Glu Asn Tyr Ser Thr Pro Pro Gln												
		290			295				300					
		Asp Leu Leu Gln Arg Gln Ala Gln Lys Tyr Lys Asp Ile Phe Thr Met												
15		305			310				315					320
		Leu Arg Lys Tyr Lys Gly Ile Val Thr Cys Val Thr Phe Trp Gly Leu												
				325				330					335	
		Lys Asp Asp Tyr Ser Trp Leu Asn Ser Ser Ser Lys Arg Asp Trp Pro												
				340				345					350	
		Leu Leu Phe Phe Asp Asp Tyr Ser Ala Lys Pro Ala Tyr Trp Ser Val												
20				355				360					365	
		Ile Glu Ala Ala Gly Ala Ser Ala Ser Pro Ser Pro Thr Val Thr Ala												
				370				375					380	
		Thr Pro Thr Pro Thr Pro Thr Pro Thr Val Thr Val Thr Ala Thr Pro												
		385			390				395					400
		Thr Pro Thr Pro Thr Gly Thr Pro Gly Thr Gly Ser Gly Leu Lys Val												
25				405				410					415	
		Leu Tyr Lys Asn Asn Glu Thr Ser Ala Ser Thr Gly Ser Ile Arg Pro												
				420				425					430	
		Trp Phe Lys Ile Val Asn Gly Gly Ser Ser Ser Val Asp Leu Ser Arg												
				435				440					445	
30		Val Lys Ile Arg Tyr Trp Tyr Thr Val Asp Gly Asp Lys Pro Gln Ser												
				450				455					460	
		Ala Val Cys Asp Trp Ala Gln Ile Gly Ala Ser Asn Val Thr Phe Asn												
		465			470				475					480
		Phe Val Lys Leu Ser Ser Gly Val Ser Gly Ala Asp Tyr Tyr Leu Glu												
				485				490					495	
35		Val Gly Phe Ser Ser Gly Ala Gly Gln Leu Gln Pro Gly Lys Asp Ala												
				500				505					510	
		Gly Asp Ile Gln Val Arg Phe Asn Lys Asn Asp Trp Ser Asn Tyr Asn												
				515				520					525	
		Gln Ala Asp Asp Trp Ser Trp Leu Gln Ser Met Thr Asp Tyr Gly Glu												
				530				535					540	
40		Asn Ala Lys Val Thr Leu Tyr Val Asp Gly Val Leu Val Trp Gly Gln												
		545			550				555					560
		Glu Pro Gly Gly Ala Thr Pro Ala Pro Thr Ala Thr Ala Thr Pro Thr												
				565				570					575	
		Pro Ile Pro Thr Ala Thr Val Thr Pro Thr Pro Thr Ala Thr Pro Thr												
				580				585					590	
45		Ser Thr Pro Arg Pro Thr Ala Thr Ala Thr Pro Thr Pro Thr Val Ser												
				595				600					605	
		Ala Thr Pro Thr Pro Ala Pro Thr Ala Ser Pro Val Gly Gly Ser Tyr												
				610				615					620	
		Trp Thr Pro Ser Glu Ser Tyr Gly Ala Leu Lys Val Trp Tyr Ala Asn												
50		625			630				635					640
		Gly Asn Leu Ser Ser Pro Thr Asn Val Leu Asn Pro Lys Ile Lys Ile												
				645				650					655	
		Glu Asn Val Gly Thr Thr Ala Val Asp Leu Ser Arg Val Lys Val Arg												
				660				665					670	
		Tyr Trp Tyr Thr Ile Asp Gly Glu Ala Thr Gln Ser Val Ser Val Ala												
55				675				680					685	

	Ser	Ser	Ile	Asn	Pro	Ala	Tyr	Ile	Asp	Val	Lys	Leu	Gly	Ala	Asn	Ala
		690					695					700				
5	Gly	Gly	Ala	Asp	Tyr	Tyr	Val	Glu	Ile	Gly	Phe	Lys	Ser	Gly	Ala	Gly
	705					710					715					720
	Val	Leu	Ala	Ala	Gly	Gln	Ser	Thr	Lys	Glu	Ile	Arg	Leu	Ser	Ile	Gln
					725					730						735
	Lys	Gly	Ser	Gly	Ser	Tyr	Asn	Gln	Ser	Asn	Asp	Tyr	Ser	Val	Arg	Ser
				740					745					750		
10	Ala	Thr	Gly	Tyr	Ile	Glu	Asn	Glu	Lys	Val	Thr	Gly	Tyr	Ile	Asp	Asp
			755					760						765		
	Val	Leu	Val	Trp	Gly	Arg	Glu	Pro	Ser	Arg	Asn	Ala	Gln	Ile	Lys	Val
		770					775					780				
	Trp	Tyr	Ala	Asn	Gly	Asn	Leu	Ser	Ser	Pro	Thr	Asn	Val	Leu	Asn	Pro
	785					790					795					800
15	Lys	Ile	Lys	Ile	Glu	Asn	Val	Gly	Thr	Thr	Ala	Val	Asp	Leu	Ser	Arg
					805					810						815
	Val	Lys	Val	Arg	Tyr	Trp	Tyr	Thr	Ile	Asp	Gly	Glu	Ala	Thr	Gln	Ser
				820					825						830	
	Val	Ser	Val	Thr	Ser	Ser	Ile	Asn	Pro	Ala	Tyr	Ile	Asp	Val	Lys	Phe
			835					840					845			
20	Val	Lys	Leu	Gly	Ala	Asn	Ala	Gly	Gly	Ala	Asp	Tyr	Tyr	Val	Glu	Ile
			850				855					860				
	Gly	Phe	Lys	Ser	Gly	Ala	Gly	Val	Leu	Ala	Ala	Gly	Gln	Ser	Thr	Lys
	865					870					875					880
	Glu	Ile	Arg	Leu	Ser	Ile	Gln	Lys	Gly	Ser	Gly	Ser	Tyr	Asn	Gln	Ser
					885					890					895	
25	Asn	Asp	Tyr	Ser	Ile	Arg	Ser	Ala	Asn	Ser	Tyr	Ile	Glu	Asn	Glu	Lys
				900					905						910	
	Val	Thr	Gly	Tyr	Ile	Asp	Gly	Ala	Ile	Val	Trp	Gly	Arg	Glu	Pro	Ser
			915				920						925			
	Arg	Gly	Thr	Lys	Pro	Ala	Gly	Val	Val	Thr	Pro	Thr	Pro	Ala	Pro	Thr
		930					935					940				
30	Pro	Thr	Ser	Thr	Pro	Thr	Pro	Ile	Pro	Thr	Thr	Thr	Pro	Thr	Pro	Thr
	945					950					955					960
	Pro	Thr	Pro	Thr	Val	Thr	Val	Thr	Pro	Thr	Ser	Thr	Pro	Thr	Pro	Val
					965					970					975	
	Ser	Ser	Ser	Thr	Pro	Thr	Pro	Thr	Ala	Thr	Pro	Thr	Pro	Thr	Pro	Ser
				980					985					990		
35	Ile	Thr	Ile	Thr	Pro	Ala	Pro	Thr	Ala	Thr	Pro	Thr	Pro	Thr	Pro	Ser
			995					1000					1005			
	Val	Thr	Asp	Asp	Thr	Asn	Asp	Trp	Leu	Phe	Ala	Ala	Gln	Gly	Asn	Lys
		1010					1015					1020				
40	Ile	Val	Asp	Lys	Asp	Gly	Lys	Pro	Val	Trp	Leu	Thr	Gly	Val	Asn	Trp
	1025					1030					1035					104
	Phe	Gly	Phe	Asn	Thr	Gly	Thr	Asn	Val	Phe	Asp	Gly	Val	Trp	Ser	Cys
					1045					1050					1055	
	Asn	Leu	Lys	Ser	Ala	Leu	Ala	Glu	Ile	Ala	Asn	Arg	Gly	Phe	Asn	Leu
				1060					1065					1070		
45	Leu	Arg	Val	Pro	Ile	Ser	Ala	Glu	Leu	Ile	Leu	Asn	Trp	Ser	Lys	Gly
			1075					1080						1085		
	Ile	Tyr	Pro	Lys	Pro	Asn	Ile	Asn	Tyr	Tyr	Val	Asn	Pro	Glu	Leu	Glu
		1090					1095					1100				
	Gly	Leu	Thr	Ser	Leu	Glu	Val	Phe	Asp	Phe	Val	Val	Lys	Thr	Cys	Lys
	1105					1110					1115					112
50	Glu	Val	Gly	Leu	Lys	Ile	Met	Leu	Asp	Ile	His	Ser	Ala	Lys	Thr	Asp
					1125						1130				1135	
	Ala	Met	Gly	His	Ile	Tyr	Pro	Val	Trp	Tyr	Thr	Asp	Thr	Ile	Thr	Pro
				1140					1145					1150		
	Glu	Asp	Tyr	Tyr	Lys	Ala	Cys	Glu	Trp	Ile	Thr	Glu	Arg	Tyr	Lys	Asn
		1155						1160					1165			
55	Asp	Asp	Thr	Ile	Val	Ala	Phe	Asp	Leu	Lys	Asn	Glu	Pro	His	Gly	Lys



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	1170		1175		1180
	Pro Trp Gln Asp Ser Val Phe Ala Lys Trp Asp Asn Ser Thr Asp Ile				
	1185		1190		1195
5	Asn Asn Trp Lys Tyr Ala Ala Glu Thr Cys Ala Lys Arg Ile Leu Ala				120
		1205		1210	1215
	Lys Asn Pro Asn Met Leu Ile Val Ile Glu Gly Ile Glu Ala Tyr Pro		1225		1230
		1220			
	Lys Asp Asp Val Thr Trp Thr Ser Lys Ser Ser Ser Asp Tyr Tyr Ser		1240		1245
		1235			
10	Thr Trp Trp Gly Gly Asn Leu Arg Gly Val Lys Lys Tyr Pro Ile Asn				
		1250		1255	1260
	Leu Gly Gln Tyr Gln Asn Lys Val Val Tyr Ser Pro His Asp Tyr Gly				
	1265		1270		1275
	Pro Leu Val Tyr Gln Gln Pro Trp Phe Tyr Pro Gly Phe Thr Lys Asp				128
		1285		1290	1295
15	Thr Leu Tyr Asn Asp Cys Trp Arg Asp Asn Trp Thr Tyr Ile Met Asp				
		1300		1305	1310
	Asn Gly Ile Ala Pro Leu Leu Ile Gly Glu Trp Gly Gly Tyr Leu Asp				
		1315		1320	1325
	Gly Gly Asp Asn Glu Lys Trp Met Thr Tyr Leu Arg Asp Tyr Ile Ile				
		1330		1335	1340
20	Glu Asn His Ile His His Thr Phe Trp Cys Tyr Asn Ala Asn Ser Gly				
	1345		1350		1355
	Asp Thr Gly Gly Leu Val Gly Tyr Asp Phe Ser Thr Trp Asp Glu Gln				136
		1365		1370	1375
	Lys Tyr Asn Phe Leu Lys Pro Ala Leu Trp Gln Asp Ser Lys Gly Arg				
25		1380		1385	1390
	Phe Val Gly Leu Asp His Lys Arg Pro Leu Gly Thr Asn Gly Lys Asn				
		1395		1400	1405
	Ile Asn Ile Thr Ile Tyr Tyr Gln Asn Gly Glu Lys Pro Pro Val Pro				
		1410		1415	1420
30	Lys Asn				
	1425				

(2) INFORMATION FOR SEQ ID NO:44:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1751 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:44:

	Met Gln Glu Met Lys Ala Ile Lys Arg Val Val Ser Ile Thr Ala Leu		
	1	5	10
45	Leu Val Leu Thr Leu Ser Leu Cys Phe Pro Gly Ile Met Pro Val Lys		
		20	25
	Ala Tyr Ala Gly Gly Thr Tyr Asn Tyr Gly Glu Ala Leu Gln Lys Thr		30
		35	40
	Ile Met Phe Tyr Glu Phe Gln Met Ser Gly Lys Leu Pro Ser Trp Val		45
		50	55
50	Arg Asn Asn Trp Arg Gly Asp Ser Gly Leu Asp Asp Gly Lys Asp Val		60
	65	70	75
	Gly Leu Asp Leu Thr Gly Gly Trp His Asp Ala Gly Asp His Val Lys		80
		85	90
	Phe Asn Leu Pro Met Ser Tyr Ser Ala Ser Met Leu Gly Trp Ala Val		95
		100	105
55	Tyr Glu Tyr Lys Asp Ala Phe Val Lys Ser Lys Gln Leu Glu His Ile		110

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5																	
10																	
15																	
20																	
25																	
30																	
35																	
40																	
45																	
50																	

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	Gly	Leu	Thr	Ser	Gln	Leu	Glu	Lys	Asn	Lys	Tyr	Ile	Ala	Ala	Tyr	Asp
	610						615					620				
5	Asn	Asn	Asn	Leu	Val	Trp	Gly	Leu	Glu	Pro	Gly	Ala	Ala	Thr	Ser	Thr
	625					630					635					640
	Pro	Ala	Pro	Thr	Ser	Thr	Pro	Thr	Pro	Thr	Pro	Thr	Pro	Thr	Pro	Thr
					645					650						655
	Val	Thr	Ala	Thr	Pro	Thr	Pro	Thr	Pro	Thr	Pro	Thr	Pro	Thr	Gly	Ser
					660					665					670	
10	Pro	Gly	Thr	Gly	Ser	Gly	Val	Lys	Val	Leu	Tyr	Lys	Asn	Asn	Glu	Thr
		675						680					685			
	Ser	Ala	Ser	Thr	Gly	Ser	Ile	Arg	Pro	Trp	Phe	Lys	Ile	Val	Asn	Gly
	690						695					700				
	Gly	Ser	Ser	Ser	Val	Asp	Leu	Ser	Arg	Val	Lys	Ile	Arg	Tyr	Trp	Tyr
	705					710					715					720
15	Thr	Val	Asp	Gly	Asp	Lys	Pro	Gln	Ser	Ala	Val	Cys	Asp	Trp	Ala	Gln
					725					730						735
	Ile	Gly	Ala	Ser	Asn	Val	Thr	Phe	Asn	Phe	Val	Lys	Leu	Ser	Ser	Gly
					740				745						750	
	Val	Ser	Gly	Ala	Asp	Tyr	Tyr	Leu	Glu	Val	Gly	Phe	Ser	Ser	Gly	Ala
		755						760					765			
20	Gly	Gln	Leu	Gln	Pro	Gly	Lys	Asp	Thr	Gly	Asp	Ile	Gln	Val	Arg	Phe
	770						775					780				
	Asn	Lys	Asn	Asp	Trp	Ser	Asn	Tyr	Asn	Gln	Ala	Asp	Asp	Trp	Ser	Trp
	785					790					795					800
	Leu	Gln	Ser	Met	Thr	Asn	Tyr	Gly	Glu	Asn	Ala	Lys	Val	Thr	Leu	Tyr
					805					810						815
25	Val	Asp	Gly	Val	Leu	Val	Trp	Gly	Gln	Glu	Pro	Gly	Gly	Ala	Thr	Pro
					820				825						830	
	Ala	Pro	Thr	Ser	Thr	Ala	Thr	Pro	Thr	Pro	Thr	Pro	Thr	Ala	Thr	Pro
			835					840						845		
	Thr	Pro	Thr	Pro	Thr	Pro	Thr	Pro	Thr	Pro	Thr	Val	Ser	Ala	Thr	Pro
	850						855					860				
30	Thr	Pro	Ala	Pro	Thr	Ala	Ser	Pro	Val	Gly	Gly	Ser	Tyr	Trp	Thr	Pro
	865					870					875					880
	Ser	Glu	Ser	Tyr	Gly	Ala	Leu	Lys	Val	Trp	Tyr	Ala	Asn	Gly	Asn	Leu
					885					890						895
	Ser	Ser	Pro	Thr	Asn	Val	Leu	Asn	Pro	Lys	Ile	Lys	Ile	Glu	Asn	Val
					900				905					910		
35	Gly	Thr	Thr	Ala	Val	Asp	Leu	Ser	Arg	Val	Lys	Val	Arg	Tyr	Trp	Tyr
		915						920					925			
	Thr	Ile	Asp	Gly	Glu	Ala	Thr	Gln	Ser	Val	Ser	Val	Ala	Ser	Ser	Ile
		930					935					940				
	Asn	Pro	Ala	Tyr	Ile	Asp	Val	Lys	Phe	Val	Lys	Leu	Gly	Ala	Asn	Ala
	945					950					955					960
40	Gly	Gly	Ala	Asp	Tyr	Tyr	Val	Glu	Ile	Gly	Phe	Lys	Ser	Gly	Ala	Gly
					965					970						975
	Val	Leu	Ala	Ala	Gly	Gln	Ser	Thr	Lys	Glu	Ile	Arg	Leu	Ser	Ile	Gln
					980				985					990		
45	Lys	Gly	Ser	Gly	Ser	Tyr	Asn	Gln	Ser	Asn	Asp	Tyr	Ser	Val	Arg	Ser
			995					1000					1005			
	Ala	Asn	Ser	Tyr	Ile	Glu	Asn	Glu	Lys	Val	Thr	Gly	Tyr	Ile	Asp	Asp
		1010					1015					1020				
	Val	Leu	Val	Trp	Gly	Arg	Glu	Pro	Gly	Arg	Asn	Ala	Gln	Ile	Lys	Val
	1025					1030					1035					104
50	Trp	Tyr	Ala	Asn	Gly	Asn	Leu	Gly	Ser	Met	Thr	Asn	Val	Leu	Asn	Pro
					1045					1050					1055	
	Lys	Ile	Lys	Ile	Glu	Asn	Val	Gly	Thr	Thr	Ala	Val	Asp	Leu	Ser	Arg
					1060				1065					1070		
	Val	Lys	Val	Arg	Tyr	Trp	Tyr	Thr	Ile	Asp	Gly	Glu	Ala	Thr	Gln	Ser
			1075					1080					1085			
55	Val	Ser	Val	Thr	Ser	Ser	Ile	Asn	Pro	Ala	Tyr	Ile	Asp	Val	Lys	Phe

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	1090		1095		1100
	Val Lys Leu Gly Ala Asn Ala Gly Gly Ala Asp Tyr Tyr Val Glu Ile				
	1105		1110		1115
5	Gly Phe Lys Ser Gly Ala Gly Val Leu Ala Ala Gly Gln Ser Thr Lys				112
		1125		1130	1135
	Glu Ile Arg Leu Ser Ile Gln Lys Gly Ser Gly Ser Tyr Asn Gln Ser				
		1140		1145	1150
	Asn Asp Tyr Ser Val Arg Ser Ala Thr Gly Tyr Ile Glu Asn Glu Lys				
		1155		1160	1165
10	Val Thr Gly Tyr Ile Asp Gly Ala Ile Val Trp Gly Arg Glu Pro Ser				
		1170		1175	1180
	Arg Gly Thr Lys Pro Ala Gly Gly Val Thr Pro Thr Pro Ala Pro Thr				
	1185		1190		1195
	Pro Thr Ser Thr Pro Thr Pro Thr Pro Thr Thr Thr Pro Thr Pro Thr				
		1205		1210	1215
15	Pro Thr Val Thr Val Thr Pro Thr Pro Thr Pro Ala Val Thr Pro Asp				
		1220		1225	1230
	Val Lys Ile Ser Ile Asp Thr Ser Arg Gly Arg Thr Lys Ile Ser Pro				
		1235		1240	1245
	Tyr Ile Tyr Gly Ala Asn Gln Asp Ile Gln Gly Val Val His Pro Ala				
		1250		1255	1260
20	Arg Arg Leu Gly Gly Asn Arg Leu Thr Gly Tyr Asn Trp Glu Asn Asn				
	1265		1270		1275
	Met Ser Asn Ala Gly Ser Asp Trp Tyr His Ser Ser Asp Asp Tyr Met				
		1285		1290	1295
	Cys Tyr Ile Met Gly Ile Thr Gly Asn Asp Lys Asn Val Pro Ala Ala				
		1300		1305	1310
25	Val Val Ser Lys Phe His Glu Gln Ser Ile Lys Gln Asn Ala Tyr Ser				
		1315		1320	1325
	Ala Ile Thr Leu Gln Met Val Gly Tyr Val Ala Lys Asp Gly Asn Gly				
		1330		1335	1340
30	Thr Val Ser Glu Ser Glu Thr Ala Pro Ser Pro Arg Trp Ala Glu Val				
	1345		1350		1355
	Lys Phe Lys Lys Asp Gly Ala Leu Ser Leu Gln Pro Asp Val Asn Asp				
		1365		1370	1375
	Asn Tyr Val Tyr Met Asp Glu Phe Ile Asn Tyr Leu Ile Asn Lys Tyr				
		1380		1385	1390
35	Gly Arg Ser Ser Ser Ala Thr Gly Ile Lys Gly Tyr Ile Leu Asp Asn				
		1395		1400	1405
	Glu Pro Asp Leu Trp Phe Thr Thr His Pro Arg Ile His Pro Gln Lys				
		1410		1415	1420
	Val Thr Cys Ser Glu Leu Ile Asn Lys Ser Val Glu Leu Ala Lys Val				
	1425		1430		1435
40	Ile Lys Thr Leu Asp Pro Asp Ala Glu Ile Phe Gly Pro Ala Ser Tyr				
		1445		1450	1455
	Gly Phe Val Gly Tyr Leu Thr Leu Gln Asp Ala Pro Asp Trp Asn Gln				
		1460		1465	1470
	Val Lys Gly Asn His Arg Trp Phe Leu Ser Trp Tyr Leu Glu Gln Met				
		1475		1480	1485
45	Lys Lys Ala Ser Asp Ser Phe Gly Lys Arg Leu Leu Asp Val Leu Asp				
		1490		1495	1500
	Ile His Trp Tyr Pro Glu Ala Gln Val Gly Gly Val Arg Ile Cys Phe				
	1505		1510		1515
	Asp Gly Glu Asn Ser Thr Ser Arg Asp Val Ala Ile Ala Arg Met Gln				
		1525		1530	1535
50	Ala Pro Arg Thr Leu Trp Asp Pro Thr Tyr Lys Thr Thr Gln Lys Gly				
		1540		1545	1550
	Gln Ile Thr Ala Gly Glu Asn Ser Trp Ile Asn Gln Trp Phe Pro Glu				
		1555		1560	1565
	Tyr Leu Pro Leu Leu Pro Asn Ile Lys Ala Asp Ile Asp Lys Tyr Tyr				
55		1570		1575	1580

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Pro Gly Thr Lys Leu Ala Ile Thr Glu Phe Asp Tyr Gly Gly Lys Asp  
 1585 1590 1595 160  
 His Ile Ser Gly Gly Ile Ala Leu Ala Asp Val Leu Gly Ile Phe Gly  
 1605 1610 1615  
 Lys Tyr Gly Val Tyr Met Ala Ala Arg Trp Gly Asp Ser Gly Ser Tyr  
 1620 1625 1630  
 Ala Gln Ala Ala Tyr Asn Ile Tyr Leu Asn Tyr Asp Gly Lys Gly Ser  
 1635 1640 1645  
 Arg Tyr Gly Ser Thr Cys Val Ser Ala Glu Thr Thr Asp Val Glu Asn  
 1650 1655 1660  
 Met Pro Val Tyr Ala Ser Ile Glu Gly Glu Asp Asp Ser Thr Val His  
 1665 1670 1675 168  
 Ile Ile Leu Ile Asn Arg Asn Tyr Asp Arg Lys Leu Lys Ala Glu Ile  
 1685 1690 1695  
 Lys Met Asn Asn Thr Arg Val Tyr Thr Gly Gly Glu Ile Tyr Gly Phe  
 1700 1705 1710  
 Asp Ser Thr Ser Ser Gln Ile Arg Lys Met Gly Val Leu Ser Asn Ile  
 1715 1720 1725  
 Gln Asn Asn Thr Ile Thr Ile Glu Val Pro Asn Leu Thr Val Tyr His  
 1730 1735 1740  
 Ile Val Leu Thr Ser Ser Lys  
 1745 1750

(2) INFORMATION FOR SEQ ID NO:45:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 18 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:45:

GCCTGGTATG CAATATAC

18

(2) INFORMATION FOR SEQ ID NO:46:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 2029 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:46:

ATGGGAAGTG	GTGTGAAGGT	ACTGTACAAG	AACAATGAGA	CAAGTGCGAG	CACAGGTTCT	60
ATAAGGCCGT	GGTTTAAGAT	AGTGAATGGA	GGCAGCAGCA	GTGTTGATCT	TAGCAGGGTT	120
AAGATAAGAT	ACTGGTACAC	AGTGGATGGT	GACAAGCCAC	AGAGTGCGGT	ATGTGACTGG	180
GCACAGATAG	GGGCAAGCAA	TGTGACATTC	AATTTTGTGA	AGCTTAGCAG	CGGAGTGAGT	240
GGAGCGGATT	ATTACCTGGA	GGTAGGATTT	AGCAGTGGAG	CTGGGCAGTT	GCAGCCTGGT	300
AAGGACACAG	GGGATATACA	GGTAAGGTTT	AACAAGAATG	ACTGGAGCAA	TTACAATCAG	360
GCAGACGACT	GGTCATGGTT	GCAGAGCATG	ACGAATTATG	GAGAGAATGC	GAAGGTGACG	420
CTGTATGTAG	ATGGTGTTC	GGTATGGGGG	CAGGAGCCGG	GAGGAGCGGT	GACCCCAACT	480
TCTACACCCA	CACCGTTTC	ATCATCCACT	CCTACACCAA	CAGCAACGCC	AACACCTACA	540
CCTTCTATCA	CGATAACACC	AGCGCCAACT	GCAACACCCA	CTCCGACTCC	TTCTGTCACA	600
GATGATACAA	ATGATGATTG	GTTATTTGCG	CAGGGTAACA	AAATAGTCGA	CAAGGATGGC	660
AAACCTGTAT	GGTTAACAGG	AGTTAATTGG	TTTGGATTTA	ATACAGGAAC	GAATGTGTTT	720
GATGGTGTGT	GGAGTTGTAA	TCTTAAAGT	GCATTAGCTG	AGATTGCAAA	CAGAGGATTT	780

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AATTTGCTAA GAGTACCGAT TTCAGCAGAG CTGATTTTGA ATTGGTCGAA AGGAATTTAT 840
CCAAAACCAA ATATCAATTA TTATGTTAAC CCTGAGTTAG AAGGTCCTGAC GAGTTTATAG 900
GTATTTGATT TTGTAGTAAA AACATGCAAA GAAGTTGGAC TGAAAATTAT GTTGGATATT 960
5 CATAGTGCAA AAACGTATGC GATGGGGCAT ATATATCCGG TATGGTATAC AGATACTATA 1020
ACGCCAGAAG ATTATTATAA AGCATGTGAA TGGATCACAG AGAGATATAA AAATGATGAT 1080
ACAATTGTAG CATTTGATTT GAAGAATGAG CCACATGGTA AACCATGGCA AGATAGTGTT 1140
TTTGCAAAAT GGGACAATTC AACAGATATT AACAACTGGA AATATGCAGC TGAGACCTGT 1200
GCGAAGAGAA TACTTGCAAA AAATCCAAAC ATGTTAATAG TAATTGAAGG AATAGAAGCT 1260
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CCGGCTGCTA ACAAAGCCCG AAAGGAAGCT GAGTTGGCTG CTGCCACCGC TGAGCAATAA 1920
CTAGCATAAC CCCTTGGGGC CTCTAAACGG GTCTTGAGGG GTTTTTTGCT GAAAGGAGGA 1980
20 ACTATATCCG GATATCCACA GGACGGGTGT GGTGCCCATG ATCGCGTAG 2029

```

## (2) INFORMATION FOR SEQ ID NO:47:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 616 amino acids  
 (B) TYPE: amino acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: protein

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:47:

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Met Gly Ser Gly Val Lys Val Leu Tyr Lys Asn Asn Glu Thr Ser Ala
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20     25     30
35 Ser Ser Val Asp Leu Ser Arg Val Lys Ile Arg Tyr Trp Tyr Thr Val
35     40     45
Asp Gly Asp Lys Pro Gln Ser Ala Val Cys Asp Trp Ala Gln Ile Gly
50     55     60
40 Ala Ser Asn Val Thr Phe Asn Phe Val Lys Leu Ser Ser Gly Val Ser
65     70     75     80
Gly Ala Asp Tyr Tyr Leu Glu Val Gly Phe Ser Ser Gly Ala Gly Gln
85     90     95
Leu Gln Pro Gly Lys Asp Thr Gly Asp Ile Gln Val Arg Phe Asn Lys
100    105    110
45 Asn Asp Trp Ser Asn Tyr Asn Gln Ala Asp Asp Trp Ser Trp Leu Gln
115    120    125
Ser Met Thr Asn Tyr Gly Glu Asn Ala Lys Val Thr Leu Tyr Val Asp
130    135    140
Gly Val Leu Val Trp Gly Gln Glu Pro Gly Gly Ala Val Thr Pro Thr
145    150    155    160
50 Ser Thr Pro Thr Pro Val Ser Ser Ser Thr Pro Thr Pro Thr Ala Thr
165    170    175
Pro Thr Pro Thr Pro Ser Ile Thr Ile Thr Pro Ala Pro Thr Ala Thr
180    185    190
Pro Thr Pro Thr Pro Ser Val Thr Asp Asp Thr Asn Asp Asp Trp Leu
195    200    205
55 Phe Ala Gln Gly Asn Lys Ile Val Asp Lys Asp Gly Lys Pro Val Trp

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[illegible]

## 55 Claims

1. A DNA sequence free of native source genomic DNA and encoding a cellulase active protein comprising the (Cel B5) amino acid sequence extending from amino acid position No. A1001 through amino acid position No. P1424

or K1425 or N1426 in SEQ. ID No. 43, or the (Cel B4/5) amino acid sequence extending from amino acid position No. K635 through amino acid position No. N1426 in SEQ. ID No. 43, or the (Cel E1) amino acid sequence extending from amino acid position No. Y39 through amino acid position No. D481 in SEQ. ID No. 44, or the (Cel E1/2) amino acid sequence extending from amino acid position No. Y39 through amino acid position No. G635 in SEQ. ID No. 44, or the (Cel E1/2/3) amino acid sequence extending from amino acid position No. Y39 through amino acid position No. G812 in SEQ. ID No. 44, or the (Cel E6) amino acid sequence extending from amino acid position No. V1233 through amino acid position No. K1751 in SEQ. ID No. 44, or the (stability region) amino acid sequence extending from amino acid position No. E482 through amino acid position No. G635 in SEQ. ID No. 44, or the (Cel E3/B5) amino acid sequence in SEQ. ID No. 47, or a functional equivalent of said proteins.

2. A recombinant DNA vector comprising:

- a) a DNA sequence encoding a cellulase active protein according to claim 1; and
- b) heterologous vector DNA.

3. A recombinant DNA expression vector according to claim 2 in which the vector DNA comprises promoter DNA operatively controlling expression of the DNA encoding the cellulase protein.

4. A recombinant DNA expression vector according to claim 3 in which said promoter DNA is heterologous DNA.

5. A recombinant DNA expression vector according to claim 3 in which the vector DNA comprises homologous promoter DNA operatively controlling expression of the DNA encoding the cellulase protein.

6. A cell transformed with an expression vector of claim 3.

7. A recombinant cellulase active protein substantially free of proteinases of native thermophilic and alkaliphilic origin and comprising the (Cel B5) amino acid sequence extending from amino acid position No. A1001 through amino acid position No. P1424 or K1425 or N1426 in SEQ. ID No. 43, or the (Cel B4/5) amino acid sequence extending from amino acid position No. K635 through amino acid position No. N1426 in SEQ. ID No. 43, or the (Cel E1) amino acid sequence extending from amino acid position No. Y39 through amino acid position No. D481 in SEQ. ID No. 44, or the (Cel E1/2) amino acid sequence extending from amino acid position No. Y39 through amino acid position No. G635 in SEQ. ID No. 44, or the (Cel E1/2/3) amino acid sequence extending from amino acid position No. Y39 through amino acid position No. G812 in SEQ. ID No. 44, or the (Cel E6) amino acid sequence extending from amino acid position No. V1233 through amino acid position No. K1751 in SEQ. ID No. 44, or the (stability region) amino acid sequence extending from amino acid position No. E482 through amino acid position No. G635 in SEQ. ID No. 44, or the (Cel E3/B5) amino acid sequence in SEQ. ID No. 47, or a functional equivalent thereof.

8. A DNA sequence free of native source genomic DNA and encoding a fragment of cellulase active protein comprising the (tokcelef) nucleotide sequence of SEQ. ID No. 9, or its functional equivalent when used in the amplification of endoglucanase genes.

9. A recombinant DNA vector comprising:

- a) a DNA sequence of claim 8; and
- b) homologous or heterologous vector DNA.

10. A cell transformed with the expression vector of claim 9.

11. A laundry detergent composition comprising a cellulase active protein in an amount sufficient to confer anti-graying or anti-backstaining properties to the detergent composition, the cellulase active protein being selected from the group consisting of Cel B5, Cel B4/5, Cel E1, Cel E1/2, Cel E1/2/3, or Cel E6, or the protein (stability region) amino acid sequence extending from amino acid position No. E482 through amino acid position No. G635 in SEQ. ID No. 44, or Cel E3/B5, or a functional equivalent of said protein.

12. The method of treating cellulosic containing material to prevent or remove staining, backstaining, or graying, comprising contacting said material with an aqueous solution of laundry detergent composition containing a cellulase active protein in an amount sufficient to confer anti-staining or anti-backstaining or anti-graying properties to the



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laundry detergent, the cellulase active protein being selected from the group consisting of Cel B5, Cel B4/5, Cel E1, Cel E1/2, Cel E1/2/3, or Cel E6, or the protein (stability region) amino acid sequence extending from amino acid position No. E482 through amino acid position No. G635 in SEQ. ID No. 44, or Cel E3/B5, or a functional equivalent of said protein.

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13. A E.coli bacterium having the identifying characteristics of ATCC Accession Nos. 98523 or 98524 or a variant or mutant thereof which produces a cellulase active protein being selected from the group consisting of Cel B5, Cel B4/5, Cel E1, Cel E1/2, Cel E1/2/3, or Cel E6, or the protein (stability region) amino acid sequence extending from amino acid position No. E482 through amino acid position No. G635 in SEQ. ID No. 44, or Cel E3/B5, or a functional equivalent of said protein.

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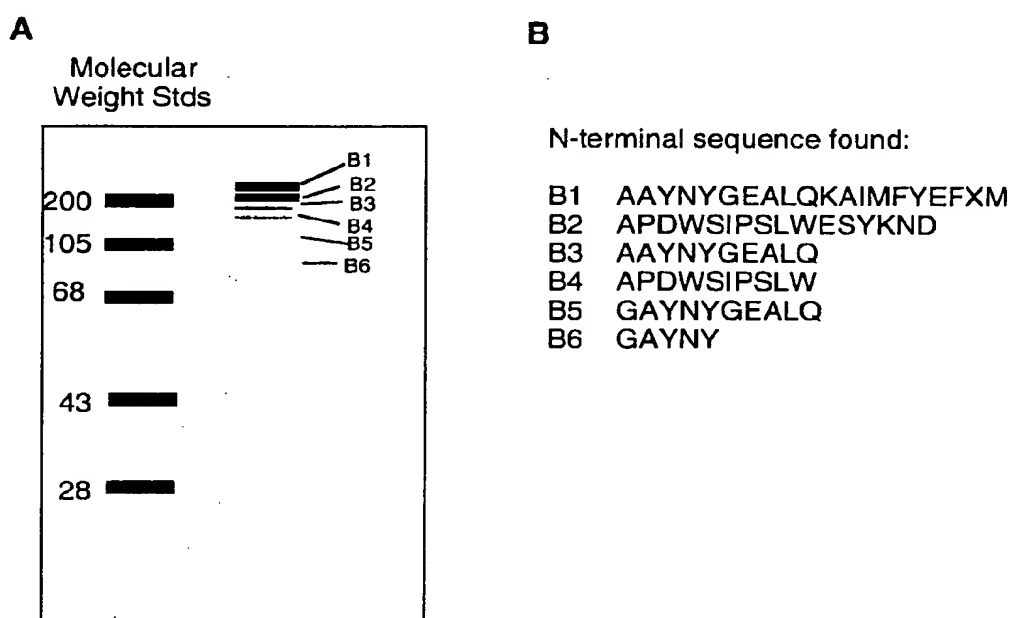
35

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**Figure 1**

A) A composite diagram of protein bands that contained cellulase activity from the Tok7B.1 supernatant purified on either S-sepharose or Q sepharose . The protein bands were designated B1 through B6 each of the designated bands was N-terminally sequenced.

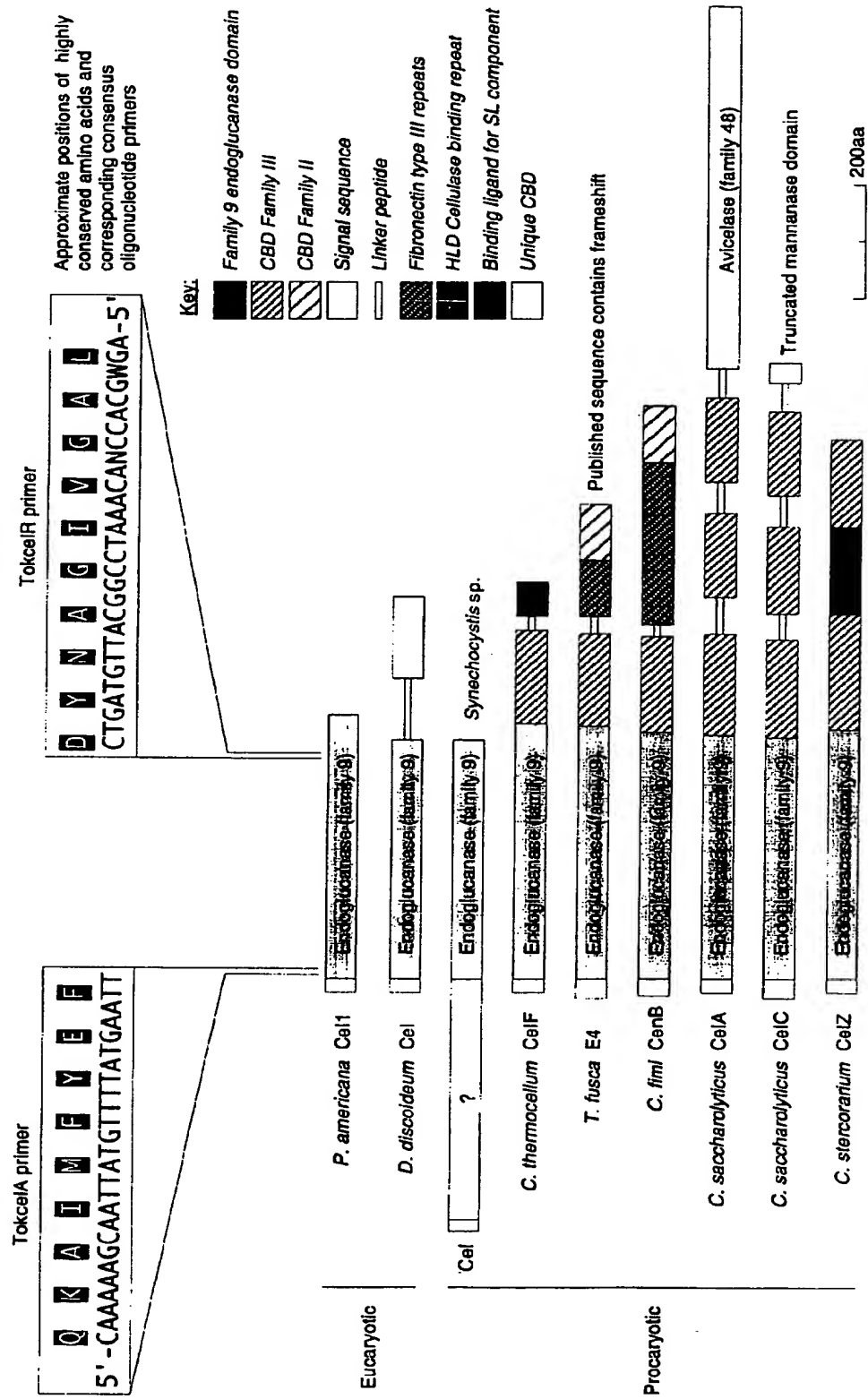
B) The N-terminal sequence found for each band is shown above. Two separate N -terminal sequences were identified corresponding to the N-terminus of the Cel E and Cel B genes shown in Figure 3.

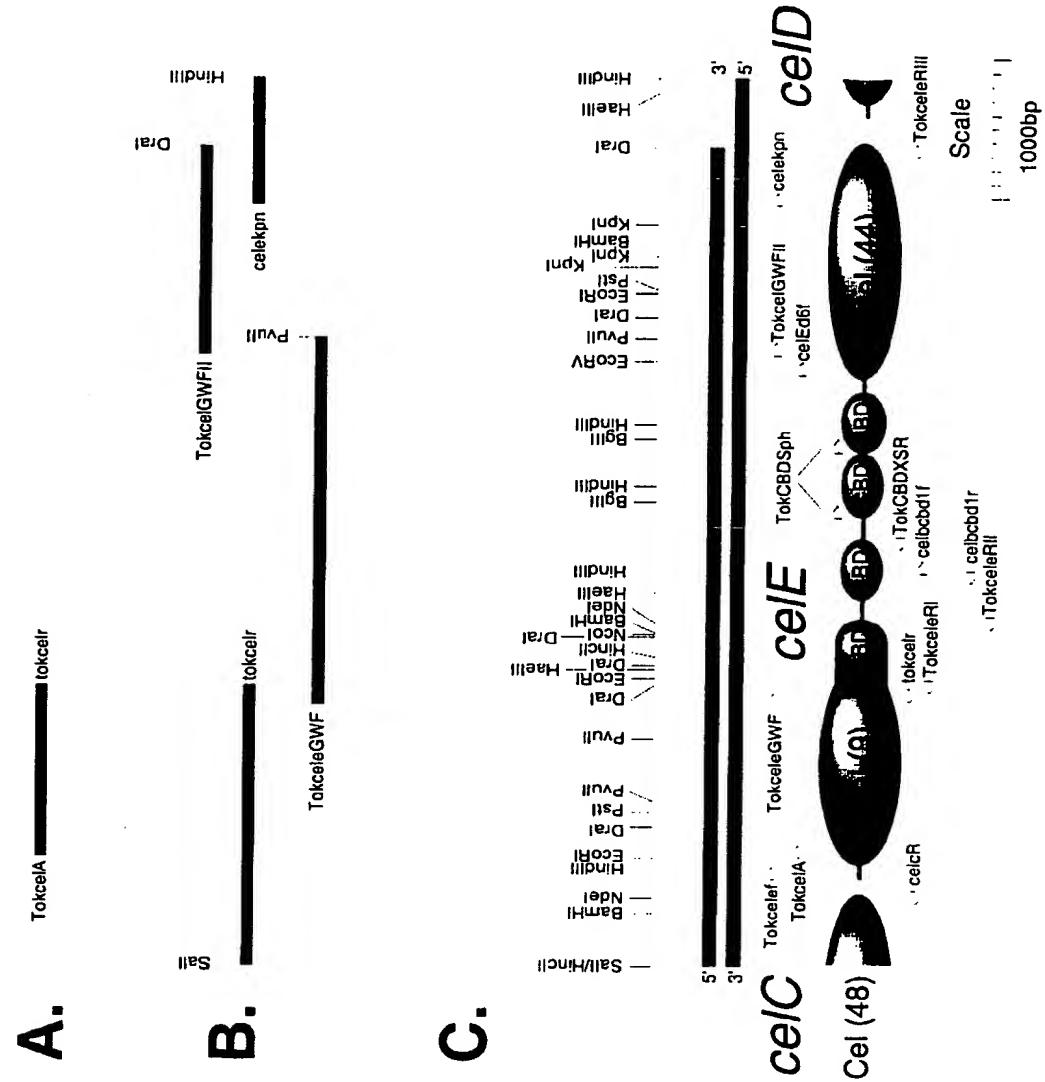
**Figure 2.**

Blast sequence homology search with the identified N-terminal peptides shows the proteins have homology with Families 9 & 10 from Glycosyl hydrolases. Areas of homology between sequenced N-termini are shown in black backgrounds with white lettering.

Peptide No.	Amino-terminal amino acid sequence	Glycosyl Hydrolase Family based on amino acid homology comparisons
B1	AAYNYGEALQKAIMFYEFXM	Glycosyl hydrolase Family 9
B3	AAYNYGEALQ	
B5	GAYNYGEALQ	
B6	GAYNY	
B2	APDWSIPSLWESKYND	Glycosyl hydrolase Family 10
B4	APDWSIPSLW	

Figure 3.





**Figure 5A.**

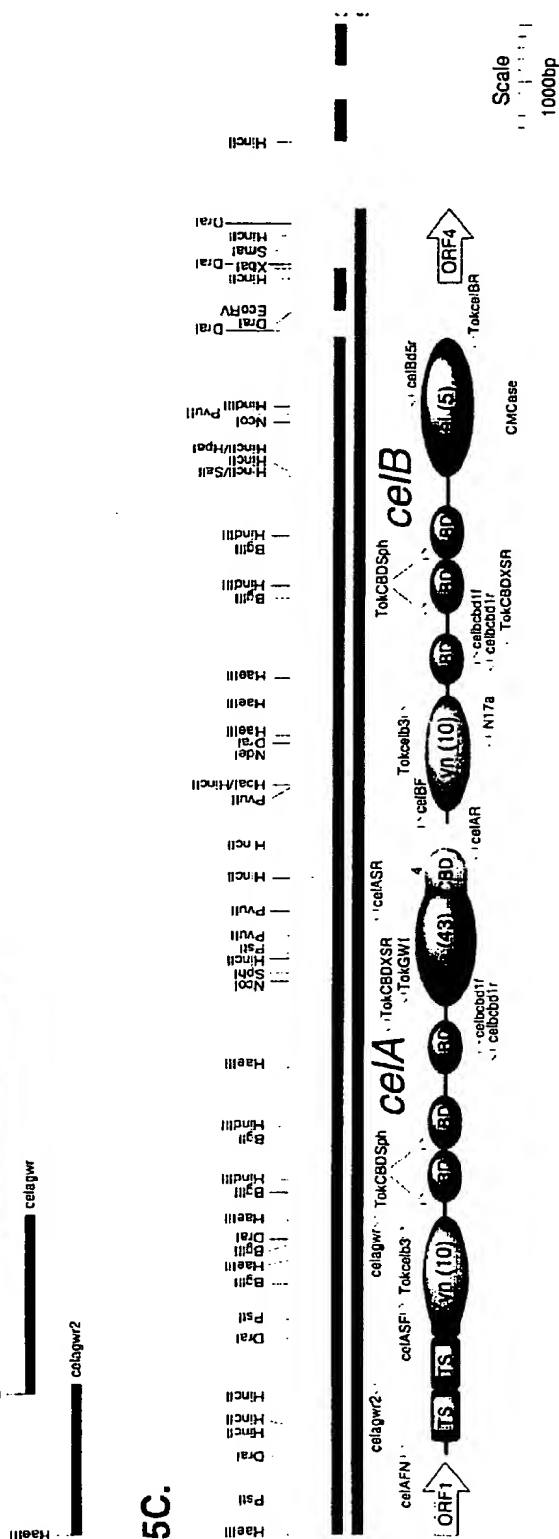
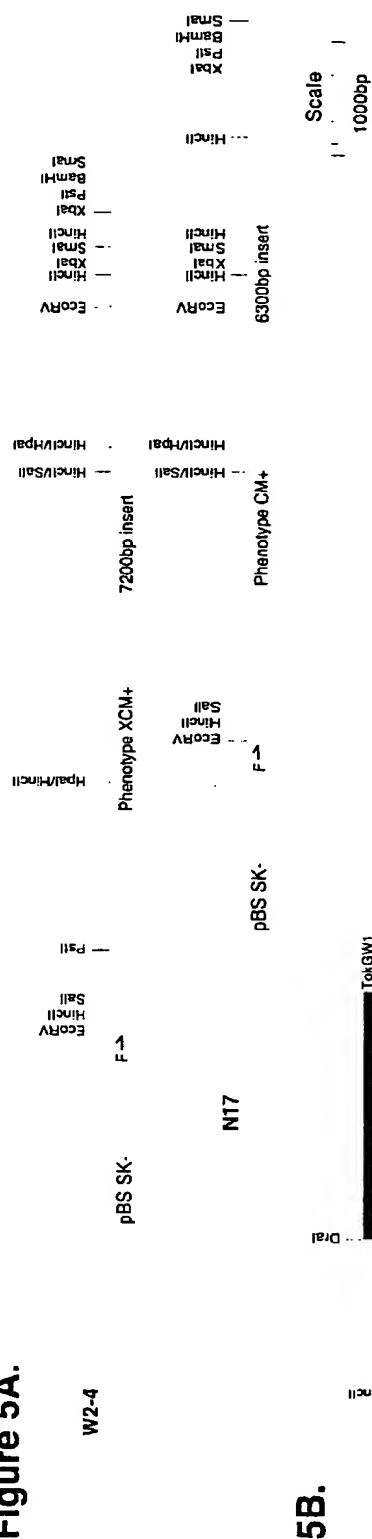


Figure 6

## Cellulase Gene Domains



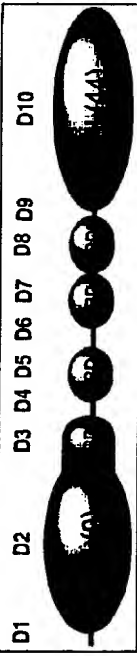
Enzyme	Protein Domain Structure	Domain	Coordinates	Function (Homology)	Reference
CelA		D1	1-33	Signal peptide	-
		D2	34-187	Thermostabilising domain	Winter
		D3	188-343	Thermostabilising domain	Winter
		D4	344-689	Endoxylanase (10)	
		D5	690-711	PT linker	Gilkes
		D6	712-877	CBD (Type III)	Tomme
		D7	878-1035	CBD (Type III)	Tomme
		D8	1036-1099	PT Linker	Gilkes
		D9	1100-1256	CBD (Type III)	Tomme
		D10	1257-1302	PT linker	Gilkes
		D11	1303-1630	Arabinofuranosidase (43)	Teo
		D12	1631-1770	CBD	Sakka
CelB		D1	1-36	Signal peptide	-
		D2	37-379	Endoxylanase (10)	
		D3	380-410	PT linker	Gilkes
		D4	411-565	CBD (Type III)	Tomme
		D5	566-616	PT linker	Gilkes
		D6	617-779	CBD (Type III)	Tomme
		D7	780-938	CBD (Type III)	Tomme
		D8	939-1007	PT linker	Gilkes
		D9	1008-1426	Endoglucanase (5)	Saul
		D10	1427-1751	Endoglucanase (44)	Gibbs
CelE		D1	1-33	Signal Peptide	-
		D2	34-472	Endoglucanase (9)	Morris
		D3	473-639	CBD (Type II)	Tomme
		D4	640-670	PT linker	Gilkes
		D5	671-830	CBD (Type III)	Tomme
		D6	831-869	PT linker	Gilkes
		D7	870-1035	CBD (Type III)	Tomme
		D8	1036-1993	CBD (Type III)	Tomme
		D9	1194-1231	PT linker	Gilkes
		D10	1232-1751	Endoglucanase (44)	Gibbs

Figure 7A.

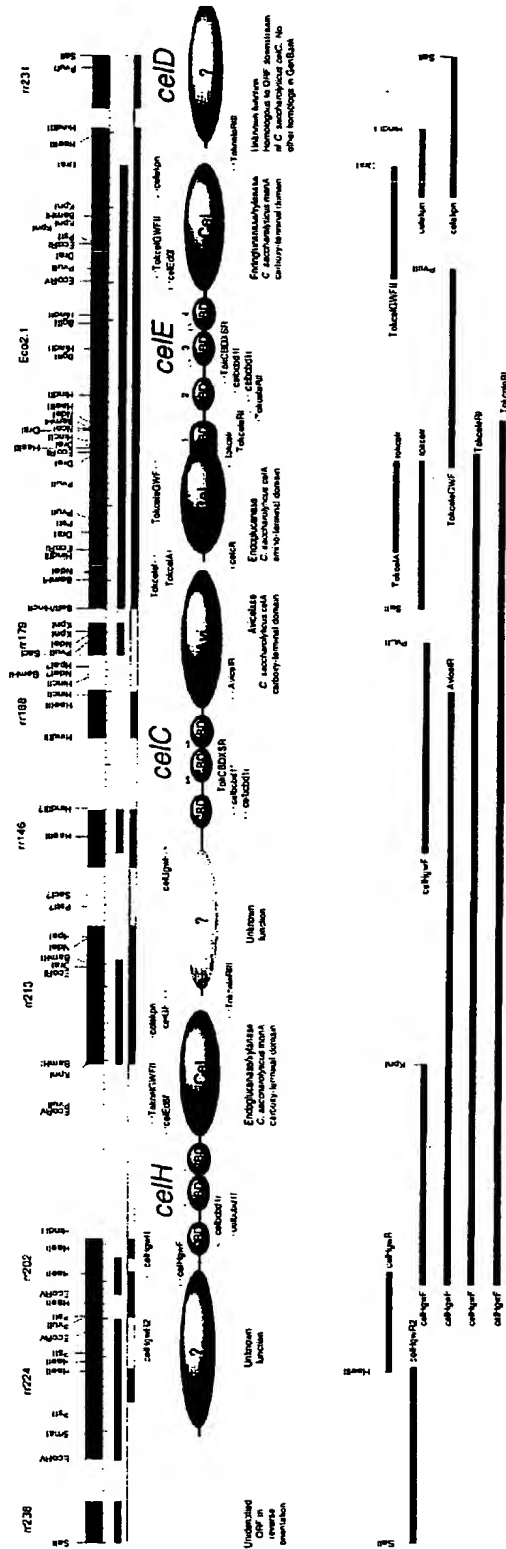
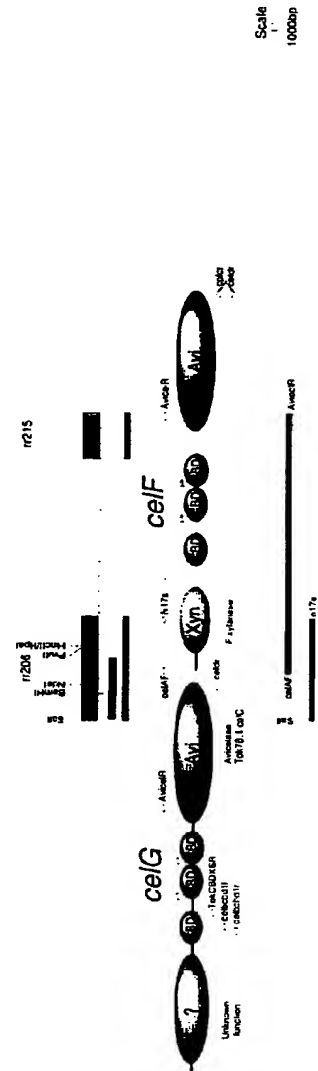
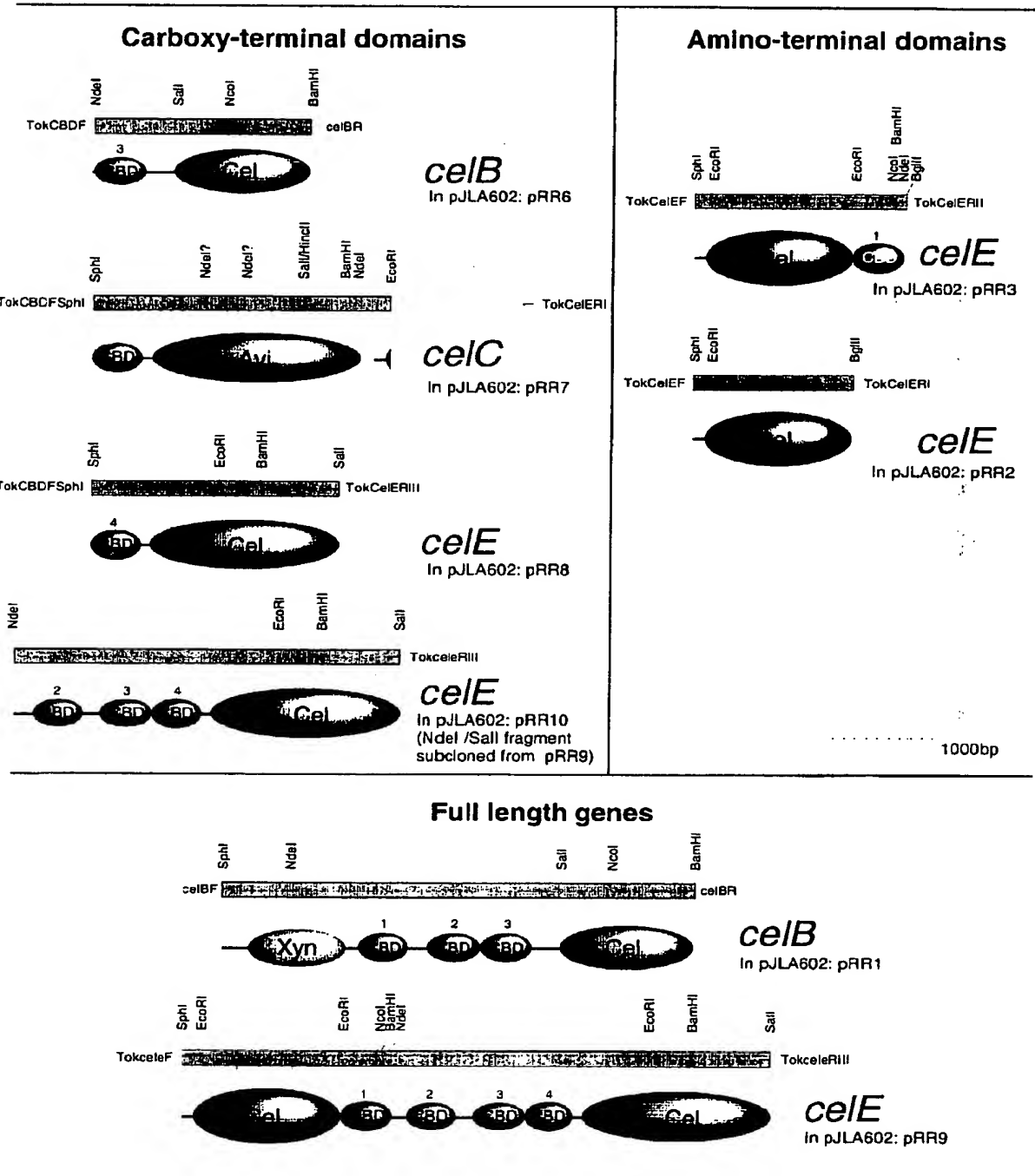


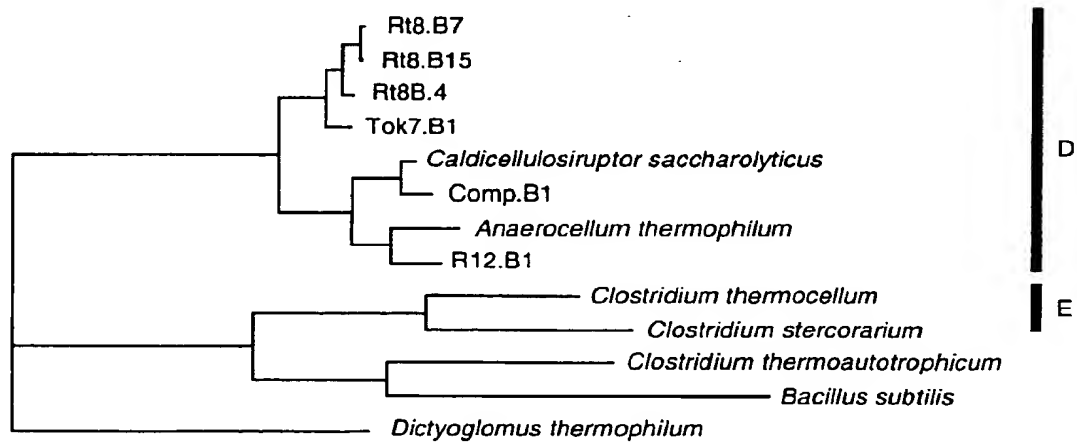
Figure 7B.





**Figure 8.*****Tok7B.1 CBD-catalytic domain PCR products expressed from pJLA602***

**Figure 9.**



**FIGURE 10**

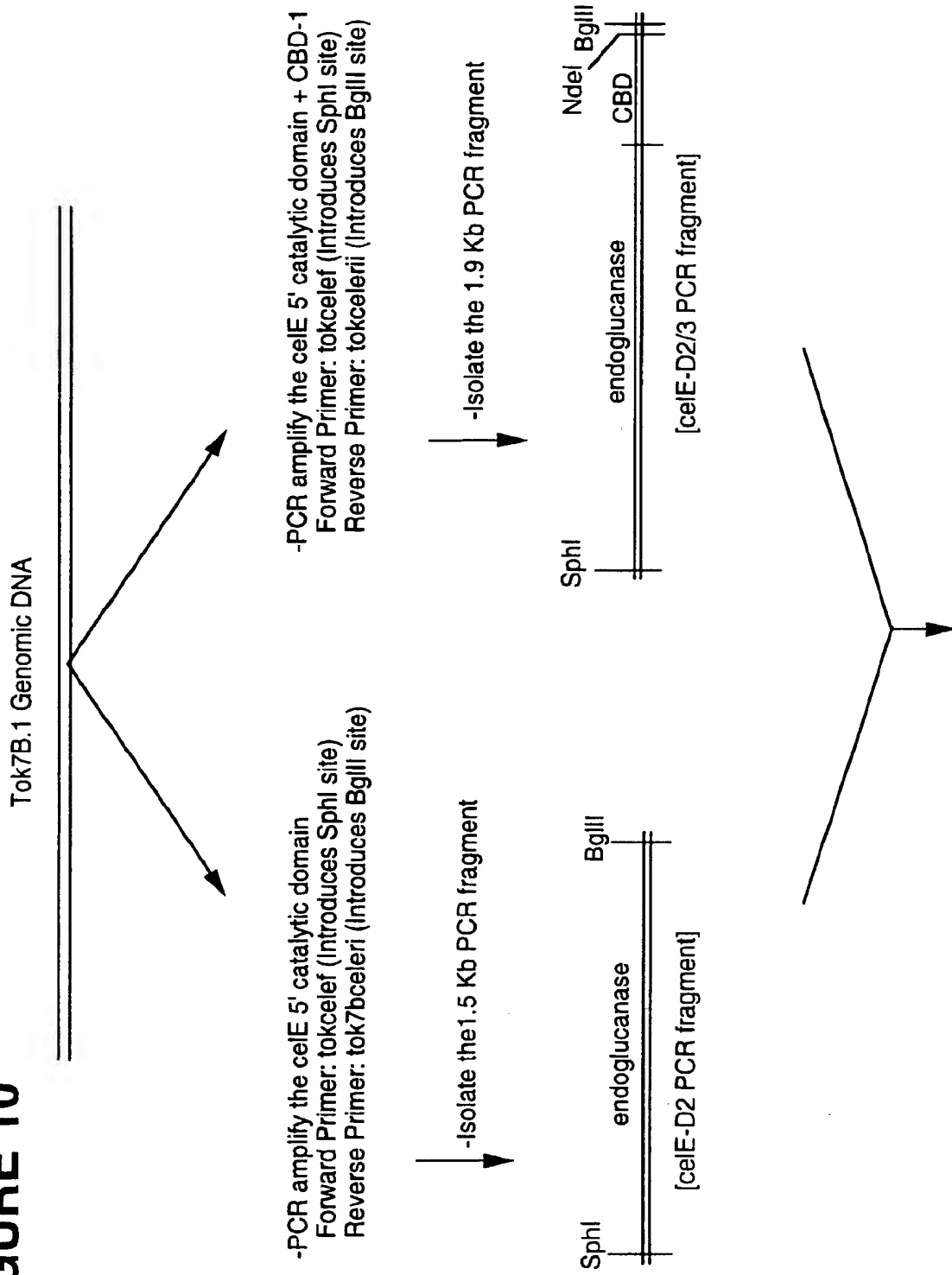
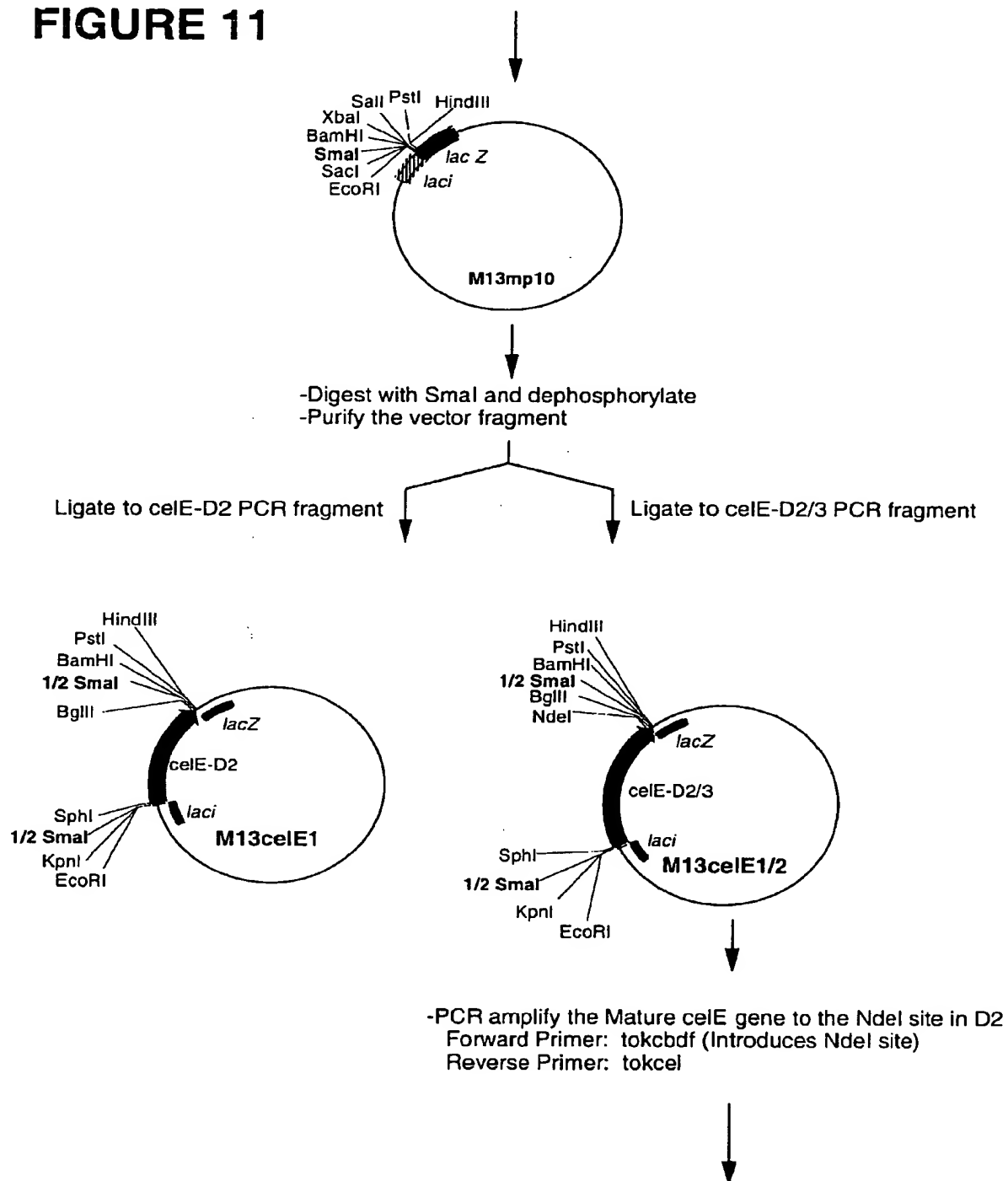


FIGURE 11



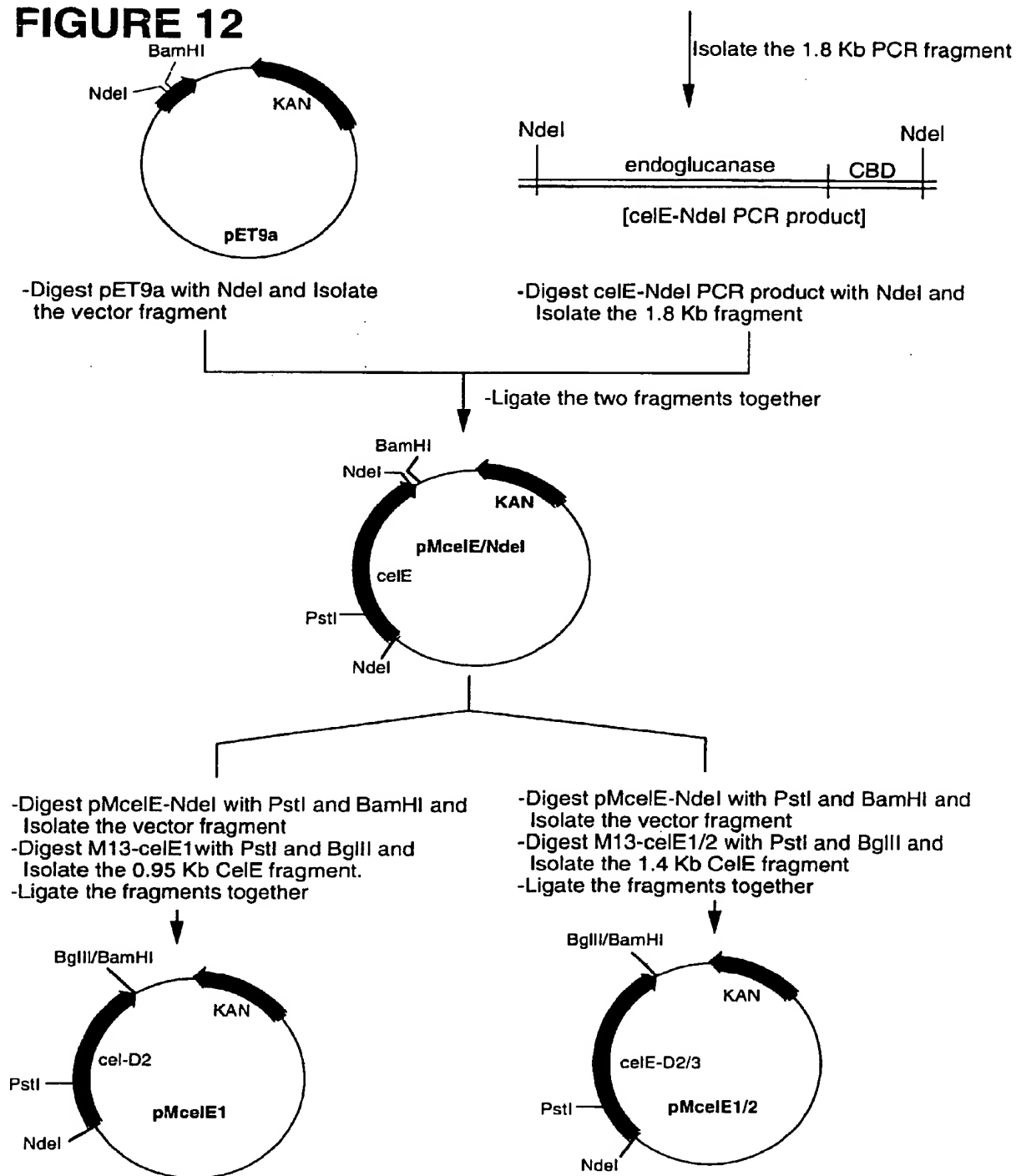
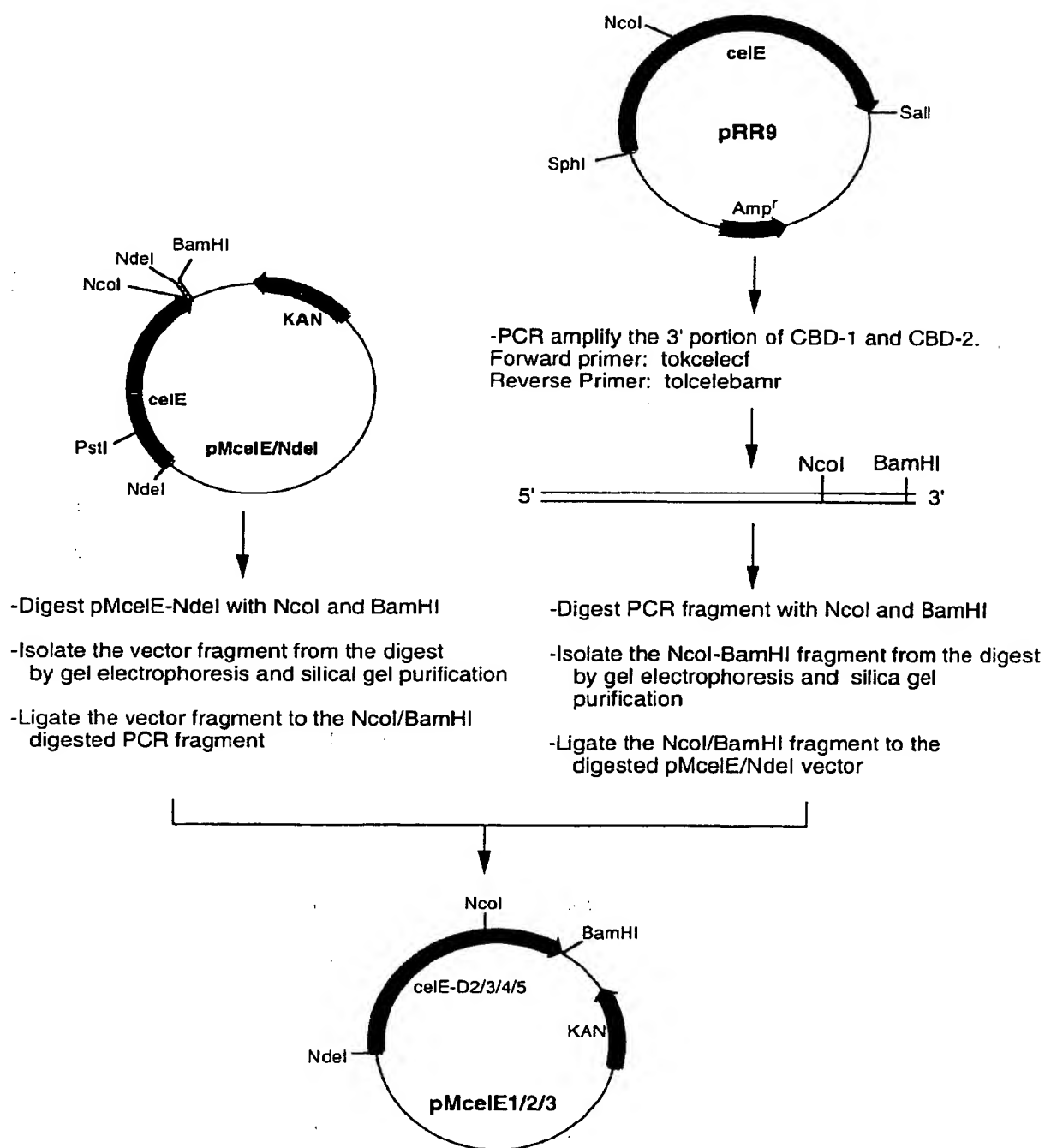
**FIGURE 12**

Figure 13



**FIGURE 14**

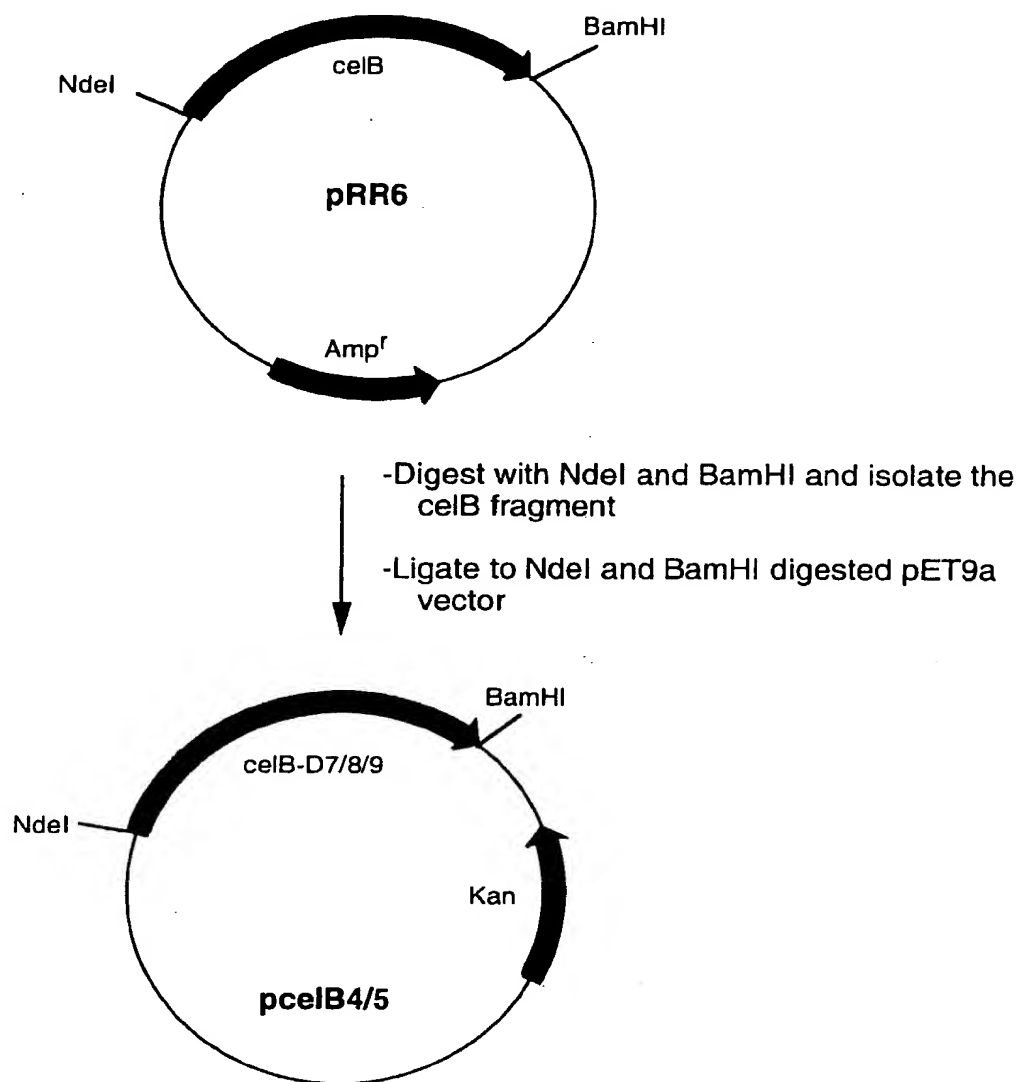
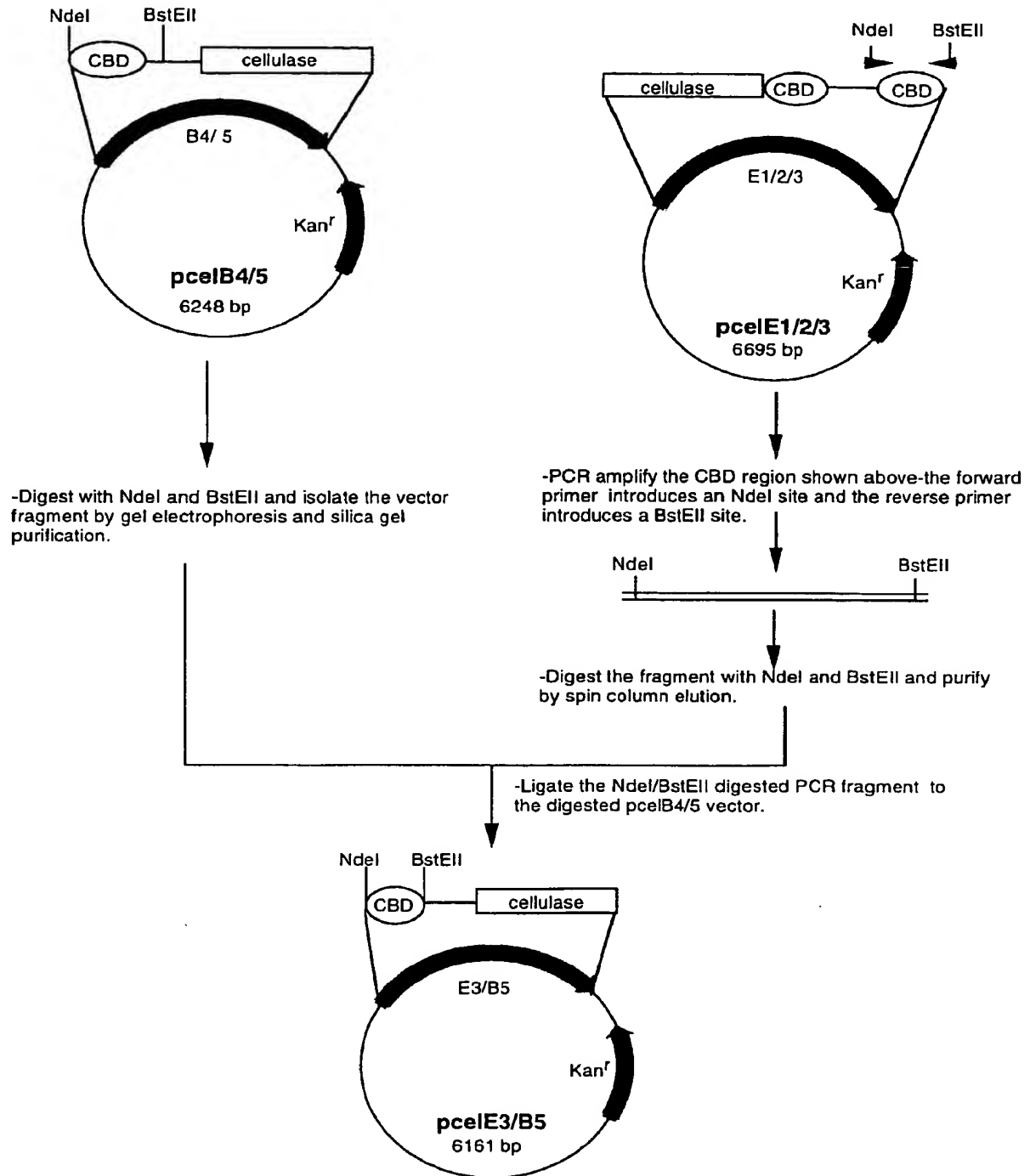


Figure 14A

## Construction of pcelE3/B5





## Figure 15.

### Sequence Analysis of the Cloned Cellulases

Cellulase Construct	N-terminal Sequence		MALDI-TOF Analysis	
	Expected	Found	Expected	Found
<b>E1</b>	<b>AAINYGEA</b>	<b>AAINYGEA</b>		
<b>E1/2</b>			<b>67,425</b>	<b>67,425 (a)</b> <b>67,245 (b)</b>
<b>B4/5</b>	<b>MKVWYANG</b>	<b>MKVWYANG (c)</b> <b>(X)PTPTPTP(T)I (d)</b>		
<b>B5</b>	<b>ATPSTPTPS</b>	<b>ATPSTPTPS</b>	<b>48.991</b>	<b>48,691(e)</b>

(a) N-terminal amino acids were changed from GT→AA in order to facilitate cloning of the protein and based on the found N-terminal sequence of the protein.

(b) N-terminal clipping of the two alanines would result in a 179 dalton decrease in the molecular weight of the protein.

(c) Sequences gave approximately equal picomolar quantities of signal.

(d) Internal cleavage site matching a site in the P-T linker region, (X) indicates that there was no amino acid was detected during the first cycle.

(e) C-terminal clipping of the the final two amino acids, lysine and asparagine, would give the correct molecular weight of 46,691.

**Table I.**

Oligonucleotide primers designed and synthesized for PCR amplification, genomic walking, and sequencing of cellulase genes from Tok7B.1.






Primer Name	Seq #	Nucleotide Sequence	Length
avicelr	21	5'- TGTATCCCATGCCGTCTT -3'	18
TokcelA	22	5'- CAAAAAGCAATTATGTTTTATGAATT -3'	26
celagwr	23	5'- TGGTGCTGGCAATGTTGAGTTGGC -3'	24
celagwr2	24	5'- TCGGTAGTGCCACTTTCAAATCCA -3'	24
celasf	25	5'- CAAAGCAGACGAATCTGT -3'	18
celasr	45	5'- GCGTGGTATGCAATATAC -3'	18
celbcd1f	26	5'- AGCTGAGCAGCGGAGTGA -3'	18
celbcd1r	27	5'- TCCACTCACTCCGCTGCT -3'	18
celbd5r	28	5'- GTTCTGATACTGTCCAAG -3'	18
celekpnr	29	5'- ACAGGCGGCGTACAACAT -3'	18
celggwf	30	5'- TTGAGGGATATGGTGACC -3'	18
celhgwf	31	5'- GAGAAACATATCCTGCAA -3'	18
celhgwr	32	5'- CCCATTTTATACCCAGGC -3'	18
celhgwr2	33	5'- TCTTGAGCAGCCATTGGA -3'	18
n17a	34	5'- GATGGCCAGTTCACGTTTATATGG -3'	24
tokcelegwf	35	5'- AGCACTGGTTGGTGGTCTGGTAG -3'	24
tokcelgwfi	36	5'- GATTGACGGGTTACAATTGGGAGAAC -3'	26
tokcelr	37	5'- AGWGCACCNACAAATCCGGCATTGTARTC -3'	29
tokgw1	38	5'- CTCCAGAATGTCATTTGTAAGATACAT -3'	27

Table II.

Oligonucleotide primers designed and synthesized for PCR amplification and directional cloning of cellulase genes from Tok7B.1. The sequence number for each primer is shown in column two.

Primer Name	Seq #	Nucleotide Sequence/ sites (Reverse text)	Engineered restriction	Length	Target Gene	Restriction site	Orientation
celar	3	5'- CCTTTATGAATTCATTACTGACTGCTA-3'		28	celA	EcoRI	Reverse
celcr	4	5'- CTTCCTCGAGAAATTCACACCCACTTTTG-3'		31	celC	XhoI, EcoRI	Reverse
celdr	5	5'- TACCCCTCGAGAAATTCATTACTCATTA-3'		30	celG	XhoI, EcoRI	Reverse
celeddf	6	5'- CTACACCCATGGTAACCCCGATGTTAA-3'		28	celE	NcoI	Forward
celff	7	5'- AAATGCTCGAGTAAAGTGAACAAGCA-3'		27	celF	XhoI	Forward
celgf	8	5'- ATGTGTCATGGCATTAAATTATTTTGTG-3'		30	celC	NcoI	Forward
tok7bcelef	9	5'- ATGCAAGGCATGCAAGCAATTAAGAGGGTTG-3'		31	celE	SphI	Forward
tok7bceleri	10	5'- TCAACAAGATCTAATCATTTGTGGGTGTTTC-3'		32	celE	BglII	Reverse
tokbdxsr	11	5'- GTGCAGCTCGAGCTCTCCGGCTCCTGCCCA-3'		34	celA-celH	XhoI, SacI	Reverse
tokcbdf	12	5'- GAGGAACGGTCAATGAAAGGTATGATGCGAATGGAA-3'		39	celA-celH	NdeI	Forward
tokcbdfsfh	13	5'- GAGGAGGAGCATGCAGATCAAGGTATGATGCGAATG-3'		38	celA-celH	SphI	Forward
tokcelb3	14	5'- TTAGCATGCTGAGGAAATACAAAG-3'		25	celB, celF	SphI	Forward
tokcelbf	15	5'- AGTTAGTGGCATGCAAAAGAGAGTTTAAAG-3'		31	celB	SphI	Forward
tokcelbr	16	5'- GAAGTATGGATCCATTTTAAATCTTTGGG-3'		31	celB	BamHI	Reverse
tokceldf	17	5'- TACAATTTAGCCATGTTAACATACATTTTAG-3'		35	celD	NcoI	Forward
tokceler3	18	5'- GCAGCAGTGTGACATTTTATCTTTAATCTAC-3'		34	celE	SalI	Reverse
tokceleri1	19	5'- GTGGATGAGATCTAACCCGGCTCTAAACCCCA-3'		32	celE	BglII	Reverse
tokcelhf	20	5'- TTGAACTTCCCATGGCAGAAATTTTACAAATGG-3'		35	celH	NcoI	Forward
tokcbdf	39	5'- GGGAAATTCATATGGCGGTATAATTACGGTGAG-3'		35	celE	NdeI	Forward
tokcelecf	41	5'- CCAGATATCACAGACAC-3'		18	celE	none	Forward
tokcelebamr	42	5'- CCTGGATCCCTACGCTCTCCGGGCTC-3'		27	celE	BamHI	Reverse
tokcel	40	5'- TATTATTATCATATGCGG-3'		15	celE	NdeI	Reverse

Table III. Gene constructs expressed in *E. coli* by a T-7 promoter.

Gene <sup>(1)</sup>	Gene Seq ID #	Protein <sup>(2)</sup> Designation	Domains Expressed	Amino Acids	Protein Seq ID #	Genetic Domains
cel E	2	CelE1		MAAY39- D481 <sup>(3)</sup>	44	D2
cel E	2	CelE1/2		MAAY39- G635 <sup>(3)</sup>	44	D2/3
cel E	2	CelE1/2/3		MAAY39- G812 <sup>(3)</sup>	44	D2/3/4/5
cel B	1	CelB4/5		MK635- N426 <sup>(4)</sup>	43	D7/8/9
cel B	1	CelB5		A1001- P424	43	D9

(1) Gene from which the clone was originally isolated.

(2) Designations of the expressed proteins.

(3) The MAA amino acids contained in the expressed proteins. The single amino acid designations reflect changes in the amino acid sequence resulting from incorporation of a NdeI restriction site at the start of the sequence.

(4) The M is preceding the gene is a result of the addition of an AUG start codon for expression in *E. coli*.

**Table IV.**

Genes constructed	Protein Construct Purified	Thermal Stability °C <sup>(1)</sup>	pH rate profile <sup>(2)</sup>	Stonewash Effect
E1	E1	55	5-9	+
E1/2	E1/2	80	4-11	+
E1/2/3	E1/2/3	ND	4-11	-
B4/5	B4/5	55	4-10	-
-	B5	70	4-10	+

(1) Thermal Stability - the highest temperature at which the protein maintains 100% of it's activity for 45 minutes at pH 7.0.

(2) The protein maintains greater than or equal to 20% of it's maximum activity at 50° C.

ND= not determined



# ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO.PCT/DK 91/00013

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.  
The members are as contained in the Swedish Patent Office EDP file on 91-03-23  
The Swedish Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP-A2- 0270974	88-06-15	JP-A- 63273474	88-11-10
		US-A- 4943532	90-07-24
		JP-A- 63273475	88-11-10
		JP-A- 63279790	88-11-15
		JP-A- 1037286	89-02-07
		JP-A- 1112982	89-05-01
		JP-A- 63141586	88-06-14
EP-A2- 0269977	88-06-08	US-A- 4962030	90-10-09
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		JP-A- 1037287	89-02-07
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		JP-A- 63240785	88-10-06
		JP-A- 63137677	88-06-09
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		US-A- 4978470	90-12-18
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34x2